Regulation of adenylyl and guanylyl cyclase in *Dictyostelium discoideum*

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Rijksuniversiteit Groningen

Regulation of adenylyl and guanylyl cyclase in *Dictyostelium discoideum*

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Romkje Valkema

geboren op 3 augustus 1961
te Spannum

Promotor: Prof. Dr. P.J.M. van Haastert

Al sa njonkenlytsen komt Piter yn’e klean

Romkje Klijnstra-Luinenburg (1897-1985)

Voorwoord

Terwijl de laatste verbeteringen uit de printer rollen, is er tijd voor bezinning. Een proefschrift is nooit het resultaat van het werk van een persoon alleen. Integendeel. Vooral tijdens de afwerking komt dit weer eens temeer naar voren.

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LIST OF ABBREVIATIONS

ACA	Aggregation stage Adenylyl Cyclase
ACG	Germination stage Adenylyl Cyclase
ANP	Atrial natriuretic peptide
βARK	β-adrenergic receptor kinase
cAMP	3′,5′-cyclic adenosine monophosphate
cAR	Cell surface cAMP receptor
cGMP	3′,5′-cyclic guanosine monophosphate
CRAC	Cytosolic regulator of adenyl cyclase
8-CPT-cAMP	8-p-chlorophenylthioadenosine 3',5'-cyclic monophosphate
DAG	Diacylglycerol
dcAMP	2′-deoxyadenosine 3′5′-monophosphate
DTT	Dithiothreitol
GC	Guanylyl cyclase
GTPγS	Guanosine 5′*O*-(thiotriphosphate)
$InsP_3$, IP_3	Inositol1,4,5-trisphosphate
kD	Kilodalton
MAP kinase	Mitogen activated protein kinase
PDE	Phosphodiesterase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	cAMP dependent protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
(Rp)-cAMPS	Adenosine 3′,5′-monophosphorothioate, (Rp)-isomer
SEM	Standard error of means

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Chapter 1

Signal transduction

Communication is a magic word in modern life; new trendy devices to contact other individuals of our species *Homo sapiens* are introduced almost every day. However, claiming communication as an invention of man would be arrogant and highly improper. Every organism, whether or not complicated, depends on communication for its well-being. The perception of food, danger or mating partners as well as sensing the cellular interior and its surroundings are indispensable for continuation of an individual organism and its species. The vast variety of life forms displays numerous strategies to maintain alive and pass on acquired hereditary material to next generations. Complex life forms like vertebrates evolved special organs to walk, swim or fly towards their food source or their mating partners. Simpler life forms like bacteria, yeasts and moulds, spending major part, if not all, of their life cycle as a unicellular organism, use asexual ways for reproduction and chasing food. In all circumstances organisms need to sense and to respond to its surroundings. For this purpose every cell holds a signalling complex that enables it to anticipate and to communicate. The sensory mechanism of organisms involves a network of signalling molecules, receptors and enzymes that, after receiving a stimulus, synthesize new messenger molecules.

CELL SURFACE RECEPTORS

Cells perceive external signals by means of receptor molecules exposed at the cell surface. Receptors transmit signals to the cellular interior to specific target molecules. Cell surface receptors are classified in different families based upon their performance. One family involves the receptors that are transmitter-gated ion channels like the nicotinic acetylcholine receptor. Binding of ligand stimulates channelling of ions across the membrane via this receptor (Raftery *et al*., 1980). The second family of cell surface receptors are the ones that modulate the activity of proteins inside the cell upon ligand binding. In this family two subfamilies can be distinguished: the G-protein coupled seven transmembrane receptors, and the subfamily of cell surface receptors with intrinsic enzyme activity. This last subfamily includes the classes of the guanylyl cyclase receptors, the protein tyrosine kinase receptors, the protein serine/threonine kinase receptors and the protein tyrosine phosphatase receptors.

The seven-transmembrane receptors

The group of seven transmembrane receptors (7TM receptor) receive extracellular stimuli, which can be chemical substances as diverse as cyclic AMP (*Dictyostelium* cAMP receptors, Saxe *et al.*, 1991), peptide hormones (yeast STE-receptors, Burkholder *et al.*, 1985; Nakayama *et al.*, 1985; Hagen *et al.*, 1986), and neurotransmitters (β-adrenergic receptor, Dixon *et al.*, 1986), or of physical nature like light pulses (rhodopsin receptor, Ovchinnikov *et al.*, 1982). Seven transmembrane receptors share a typical structure with seven stretches of 20-30 hydrophobic amino acids, which are predicted to form membrane-spanning α-helices. For bacterial rhodopsin it has been shown that the transmembrane domains form a

hydrophilic pocket for ligand binding surrounded by hydrophobic residues. It is generally accepted that other small ligands are bound to their receptors in a similar way (fig. 1) (Strader *et al.,*1989). The 7TM receptors are coupled via heterotrimeric G-proteins to various intracellular enzymes, ion channels and transporters (Birnbaumer *et al*., 1990).

Many 7TM receptors also share the ability to be desensitized, a process by which they become refractory to further stimulation after an initial response, despite the continued presence of a stimulus of constant intensity. Adaptation is characterized by rapid recovery of the responsiveness after removal of the stimulus, even in the absence of protein synthesis. Prolonged stimulation with ligand results in down regulation: reduction of the number of receptor molecules at the cell surface. Recovery of the receptor number requires de novo protein synthesis. In the β2-adrenergic receptor short-time adaptation is believed to be associated with receptor phosphorylation. Upon ligand binding, serines in the C-terminal cytosolic tail of the receptor, become phosphorylated by kinases. In the β2-adrenergic receptor this results in impairment of G-protein coupling. Ligand-induced phosphory-lation of many G protein-linked receptors is mediated by cAMP-dependent protein kinases in some instances and, more generally, by members of a recently defined family of receptor kinases (Benovic *et al*., 1989; id, 1991; Lorenz *et al*., 1991).

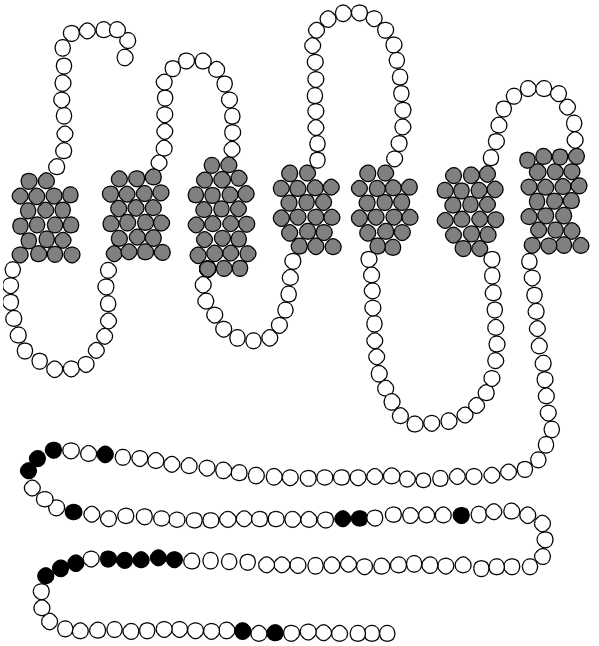

Figure 1. *Structure of a typical 7TM receptor, the Dictyostelium discoideum cAR1 receptor*
Seven stretches of the polypeptide chain are rich in hydrophobic residues (marked in grey). The hydrophobic domains that form the transmembrane pocket to which ligand binds, are connected by intra- and extracellular stretches. The phosphorylatable serine residues are found in the cytosolic tail (marked in black).

G-proteins

Intracellularly the 7TM receptor is coupled, probably by the third intracellular loop, with a molecular switch, a guanosine triphosphate-hydrolysing protein or G-protein (fig. 2). Turned on by binding GTP and off by hydrolysing GTP to GDP, G-proteins sort and amplify transmembrane signals. G-proteins are heterotrimers consisting of an α, β and γ subunit. The α subunit is a GTPase, which is associated to the plasmamembrane where it interacts

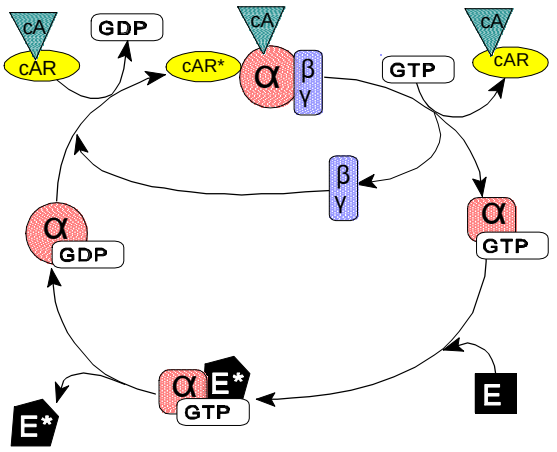

Figure 2. *Scheme of G-protein linked signal transduction*
cAR: cAMP receptor; cA: cAMP, αβγ: G-protein subunits; E: target enzyme in inactive state; E*GTPα: enzyme coupled to activating α subunit-GTP complex; E*: activated enzyme; αGDP: subunit α complexed with hydrolysed GDP.

with the βγ-complex. Ligand binding to the 7TM receptor leads to a conformation that exposes a high-affinity binding site for a G-protein in its GDP-liganded heterotrimeric form (Hamm, 1991; Dratz *et al.*, 1993). The interaction of the activated receptor and G-protein leads to the exchange of GDP for GTP on the Gα subunit resulting in dissociation of Gα from Gβγ, as well as from the activated receptor. Gα-GTP is then able to bind and regulate the appropriate effector system, and in some cases Gβγ also regulates effector activity. The system is inactivated when the intrinsic GTPase activity of the α subunit hydrolyses the GTP to GDP, the dissociated α-GDP and βγ subunits reassociate, and the system returns to its resting state.

Gα subunits are known to interact with and regulate a variety of second messenger enzymes and ionic channels, including adenylyl cyclase, PDE, PLC and K^+ and Ca^{2+} channels (De Vivo & Iyengar, 1994). However, it appears that the Gβγ subunits are also important in the regulation of many second messengers systems, alone or in concert with the activated Gα subunit (Tang & Gilman, 1991; Clapham & Neer, 1993).

Adenylyl cyclase and Phospholipase C

The 7TM-receptor-G-protein complex is coupled to effector enzymes like adenylyl cyclase and phospholipase C, that produce second messengers. Adenylyl cyclases, the enzymes that catalyse the formation of cAMP out of ATP, appear in a variety of protein structures. Most adenylyl cyclases are associated with the plasma membrane, although certain bacterial enzymes are cytosolic. Up to now, three classes of membrane-bound adenylyl cyclases have been identified. The adenylyl cyclases of *Saccharomyces cerevisiae* and *Escherichia coli* (class I) are peripheral membrane proteins, whereas the amino acid sequence of adenylyl cyclase G from *Dictyostelium* predicts one transmembrane domain (class II). A more common motif in higher eukaryotes (class III) shows two membrane spanning domains each containing six α helices, and two hydrophilic regions that are believed to be the catalytic domains (fig. 3). Because the topography of these adenylyl cyclases is homologous to various ion channels and transporters, it has been hypothesized that the function of cAMP synthesis and secretion was incorporated in one molecule (Krupinski *et al.*, 1989). However, in a *Dictyostelium* mutant with a disrupted gene for class III type adenylyl cyclase ACA, but expressing ACG (Pitt *et al.*, 1992), cAMP secretion is normal, thus ruling out the cAMP transporter function hypothesis for class III adenylyl cyclase.

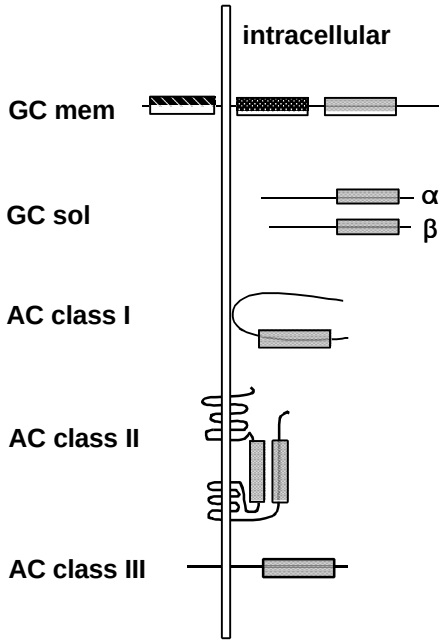

Figure 3. *Overview of predicted structures of cyclase molecules*
GC mem: guanylyl cyclase, membrane bound, receptor domain situated extracellularly, one cytosolic catalytic domain and one kinase-like domain; GC sol: soluble guanylyl cyclase, heterodimer of GC type α and β with each one catalytic domain; AC class I, membrane associated adenylyl cyclase, found in prokaryotes; AC class II, membrane bound adenylyl cyclase, amino acid composition predicts two stretches passing each six times the membrane and two putative cyclase domains (mammalian AC's and *Dictyostelium* ACA); AC class III: membrane bound adenylyl cyclase, predicted structure passes membrane once and one cyclase domain (*Dictyostelium* ACG)

Phospholipase C is found in all kind of eukaryotic organisms, varying from mammalian tissues to *Drosophila* and *Dictyostelium*. Phospholipase C proteins, are soluble proteins subdivided in three types based upon sequence conservation: β, γ and (fig. 4). All three types show two conserved amino acid domains A and B, which are considered to form the catalytic core of the enzyme. PLC type β has a large C-terminal domain adjacent to the B-domain that serves as a primary target of G-protein α subunit interaction (Park *et al.*, 1993). In PLC-γ, between the A and B domain there is an intervening sequence in which *src* homology domains SH2 and SH3 were identified (Suh *et al.*, 1988; Stahl *et al.*, 1988). SH2 and SH3 domains are involved in networks of protein-protein interaction, which are initiated after receptor stimulation. SH2 domains recognize phosphorylated tyrosine residues of specific amino acid sequences (Pawson & Gish, 1992), whereas SH3 domains may bind to proline-rich regions in several proteins (Cicchetti *et al.*,1992). In PLC- both the large C-terminal domain and the SH2/SH3 domain are absent. Instead, the N-terminal domain appears to contain a PIP_2 binding site, which is believed to conduct the protein to the membrane during hydrolysis (Rebecchi *et al.*, 1992). Furthermore all PLC's depend on the presence of Ca^{2+} for regulation of enzyme activity. In several PLC isoforms the amino acid sequence predicts the presence of an EF-hand motif, a domain found in many Ca^{2+}-modulated proteins (Cifuentes *et al.*, 1993).

The cell surface receptors with enzyme activity

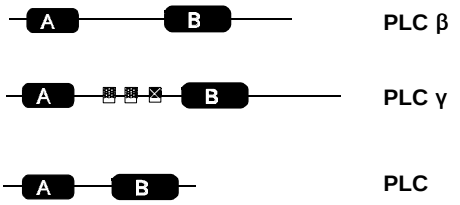

Figure 4. *Phospholipase C types* β,γ *and* δ
Common feature of the PLC types are the conserved regions, the A and B box.
In PLC-γ SH domains are found between the conserved domains, PLC has a truncated C-terminal domain

Guanylyl cyclase receptors

Guanylyl cyclases are found in both particulate and soluble fractions of cell homogenates (Kimura & Murad, 1974) (fig. 3). The group of particulate guanylyl cyclases are considered to be trans-membrane proteins and are classified as cell surface receptors. The amino acid sequences of the particulate GC's all share a more or less common motif: an extracellular domain, a short transmembrane part, and an intracellular piece that holds the catalytic activity. The protein is predicted to cross the membrane once due to a stretch of hydrophobic amino acid residues. The extracellular N-terminus, that varies from receptor to receptor, is the ligand binding domain by which GC activity can be stimulated (Bennett *et al.*, 1991). Various oligopeptides have been identified to be a ligand for membrane bound guanylyl cyclases. Eggs from sea urchin species secrete the oligopeptides speract and resact that stimulate guanylyl cyclase activity of the spermatozoa (Shimomura *et al.*, 1986). This results in sperm motility and directional movement. In mammalian cells natriuretic peptide (Hamet *et al.*, 1984; Waldman *et al.*, 1984; Winquist *et al.*, 1984), the heat-stable enterotoxin STa (Schultz *et al.*,1990), and the oligopeptide guanylin isolated from adult rat jejunum (Weigand *et al.*, 1992), were identified as potent ligands for particulate guanylyl cyclases.

In the domain that projects into the cellular interior, two conserved stretches of amino acids can be distinguished, one of which has a high homology with the catalytic domain of known protein kinases. There are no reports on kinase activity associated with guanylyl cyclases. However, deletion of this kinase domain in the ANP-receptor guanylyl cyclase resulted in a constitutively active guanylyl cyclase, an activity that was independent of ANP stimulation (Chinkers & Garbers, 1989). The domain nearest the C-terminus is the putative cyclase catalytic domain, showing high resemblance with the catalytic domains in known adenylyl cyclases.

Highly related to the particulate guanylyl cyclases are the soluble GC's. They do not belong to the cell surface receptors and will be mentioned here because of their guanylyl cyclase activity. Purified soluble guanylyl cyclase has been shown to contain an associated heme group (Gerzer *et al*., 1981) which is effectively activated by nitric oxide (NO) (Arnold *et al*., 1977; Craven & DeRubertis, 1978). DNA sequencing of the soluble guanylyl cyclases revealed four subtypes

α_1, α_2, β_1, and β_2 (Koesling *et al*., 1991; Garbers, 1992; Yuen & Garbers, 1992). The primary structure of the subtypes show stretches that have high homology with the putative catalytic domain in the particulate GC's. Experiments indicated that the active enzyme consists of an αβ-heterodimer (Garbers, 1979; Harteneck *et al.*, 1991). It is proposed that for transmembrane guanylyl cyclase also a dimer is the minimum catalytic unit, in analogy with adenylyl cyclases which contain two consensus cyclase catalytic domains in one polypeptide (Garbers *et al*., 1994). This hypothesis is confirmed by the observation that the catalytic domain of the ANP receptor when overexpressed in bacteria behaves as a dimer upon size exclusion chromatography (Thorpe *et al.,* 1991).

The activity of guanylyl cyclases is also Ca^{2+} dependent. Inhibition of guanylyl cyclase activity by Ca^{2+} was observed in retinal outer segments from vertebrate eye as well as in *Dictyostelium* cells (Koch & Stryer, 1988; Valkema & Van Haastert, 1992). In vertebrates GCAP, a ***g***uanylyl ***c***yclase-***a***ctivating ***p***rotein was identified that interacts with membrane bound guanylyl cyclase from retinal rods. GCAP binds calcium, suggesting it participates in the Ca^{2+} sensitive activa-tion of guanylyl cyclase (Palczewski *et al.*, 1994). In soluble guanylyl cyclases an indirect system of Ca^{2+} inhibition was reported: the NO synthase which provides the guanylyl cyclase activating NO, is inhibited by Ca^{2+}/calmodulin interaction (Koch *et al.*, 1994). In *Paramecium* the opposite was observed: membrane bound guanylyl cyclase is activated by submicromolar concen-trations of Ca^{2+}, in which calmodulin is involved (Klump & Schultz, 1982; Klump *et al.*, 1987).

Protein kinase receptors: tyrosine kinase and serine threonine kinase receptors

Protein phosphorylation by protein kinases is an attractive way of regulating immediate cellular responses to the environment, since phosphate groups can be added and removed easily to a variety of amino acids within polypeptide chains, thus inducing striking changes in protein enzymatic activity, localization or binding properties. Protein kinases are crucial in regulating functions such as metabolism, gene expression, cytoskeletal architecture, cell adhesion and progression through cell cycle. The eukaryotic protein kinases comprise one of the largest superfamilies of homologous proteins (reviewed in Hanks & Hunter,1995). Within this family there are now hundreds of different members which' sequences are known. They are related by virtue of their kinase domains, which consist of 250-300 amino acid residues. The kinase domains contain 12 conserved subdomains that fold into a common catalytic core structure. There are two main subdivisions within this superfamily: the protein-serine/threonine kinases and the protein-tyrosine kinases. A number of residues within the catalytic domain are highly conserved between both types of protein kinase, but the protein-tyrosine kinases and protein-serine/threonine kinases are distinguished by specific signature motifs (Hanks *et al.*, 1988). Phosphotyrosine has an elongated structure, and apparently plays a crucial role in molecular recognition and the formation of protein-protein complexes. Indeed a conserved protein module, the *src* homology 2 (SH2) domain, exists in many intracellular signalling proteins whose task it is to bind phosphotyrosine

directly (Koch *et al.*, 1991). Phosphoserine and phospho-threonine, in contrast, can in many cases be mimicked by an acidic residue, suggesting they act through a simple charge alteration (Cowley *et al.*, 1994). Their main function apparently is to modify the conformation or substrate-binding sites of enzymes whose activities are consequently altered (Barford & Johnson; 1989).

Kinases can be found as soluble proteins but also as transmembrane proteins. The transmembrane kinases act as cell surface receptors. Members of this receptor group are the insulin receptor and the growth factors receptors like the platelet derived growth factor (PDGF) receptor and the epidermal growth factor (EGF) receptor. The insulin receptor plays an important role in metabolism; binding of insulin activates amino acid and glucose transport across the plasma membrane. The EGF and PDGF receptor are involved with growth processes like cell differentiation and proliferation. In the *Drosophila* eye, development of photoreceptors requires activation of the Ras pathway by the EGF receptor (Freeman, 1994).

The molecular structure of the tyrosine kinase receptors predicts a single putative membrane spanning domain with extracellularly a ligand binding domain and intracellularly a kinase domain. The ligand binding domain of this receptor group is rather diverse, whereas the tyrosine kinase domain is the most conserved domain in this family. The insulin receptor exists in the cell membrane as a dimer, which becomes stabilized by binding of a ligand. The EGF receptor and the PDGF receptor molecules are monomers that become dimerized upon ligand binding.

Ligand binding to the tyrosine kinase receptors leads to receptor activation, which is accomplished by autophosphorylation at specific tyrosine residues. Receptor activation results besides phosphorylation of the target molecules, also in interaction with other proteins, by binding of the phosphorylated residues of the receptor with SH2 domains of the substrate molecules.

The group of transmembrane protein serine/threonine kinase receptors is a fast growing group that comprises the transforming growth factor β (TGFβ) receptor and the activin receptors. These kinases are transmembrane proteins, with extracellularly the receptor domain and intracellularly the kinase domain. Little is known about the functions of these receptor-types.

Protein tyrosine phosphatase receptors

The last group of transmembrane receptors that will be mentioned here is the group of tyrosine phosphatase receptors, which dephosphorylate tyrosinyl residues *in vivo* and take part in signal transduction and cell cycle regulation. Most of the transmembrane phosphatases contain two conserved intracellular catalytic domains; their external segments are highly variable. The structure of the transmembrane phosphatases resembles that of several integral membrane tyrosine kinases such as the epidermal growth factor: the extracellular amino-terminal domain inhabits the receptor domain, whereas the intracellular domain shows phosphatase activity. The conserved catalytic domains show homology with soluble tyrosine phosphatases (Fischer *et al.*, 1991).

A well-studied receptor phosphatase is protein CD45, an abundant

transmembrane glycoprotein from haematopoietic cells, that is involved in regulating T-cell receptor signalling. One of its substrate molecules, $p56^{lck}$, a tyrosine kinase, is thought to be regulated by tyrosine phosphorylation at the autophosphorylation site and at a negative regulatory site. Tyrosine phosphorylation at the carboxyl-terminal negative regulatory site is believed to result in an intramolecular interaction with its SH2 domain, which results in an inactive or inaccessible kinase. In the presence of CD45 this negative regulatory tyrosine residue in $p56^{lck}$ is dephosphorylated (Sieh *et al.*, 1992; Hurley *et al.*, 1993).

The reciprocal effect of phosphatases and kinases leaves room to speculate about a synergistic system, in which the kinases and phosphatases not simply act as on-off switches (Fischer *et al.*, 1991). The cyclic interconversion of these regulatory enzymes is a dynamic process, in which the steady state equilibrium between active and inactive forms varies gradually, depending on many parameters, determined by the metabolic state of the system. Because most tyrosine kinases are associated with the membrane, tyrosine phosphorylation has been regarded as a primary signal capable of affecting the state of activity of secondary enzymes, downstream. In such a cascade, receptor kinases would serve a triggering role, relying on the longer lived serine-threonine phosphorylation of the secondary enzymes to sustain the signal. Tyrosine phosphorylation would guarantee that the activity of the tyrosine kinases would be transient, thus contributing to the desensitization process.

Signal transduction in *Dictyostelium discoideum*

DEVELOPMENT IN DICTYOSTELIUM

The amoebae of the cellular slime mould *Dictyostelium discoideum* originally live separately in the upper layer of the soil, where they feed on bacteria, on spores of other fungi or by absorbing nutrients from dead organic matter. Depletion of the food source leads to aggregation of the amoeboid cells to a multicellular organism (fig. 5). Upon starvation, cells in the centre of the amoeba population emit cAMP in a pulsatory manner, that diffuses into the surroundings and induces neighbouring amoebae to migrate towards them. The moving cells in turn synthesize and secrete cAMP themselves, ensuring that the signal spreads in the area. Up to 100,000 cells are involved in the chemotactic formation of one aggregate. The tip of the aggregate rises to form a finger-like structure, which falls down on the substratum, where it moves around like a slug. The cells in the slug differentiate in two different types: prespore cells and prestalk cells. Finally the slug comes at ease and the cells reposition into the Mexican-hat shape, out of which the fruiting body is formed. The prestalk cells become the highly

vacuolized stalk cells, that carry the spore head with spore cells embedded in slime droplets. Under favourable conditions, the spores spread in the area and germinate, thus starting the whole cycle over.

During the vegetative stage of *Dictyostelium*, folic acid, a secretion product of bacteria, enables the amoeba to locate its food source. Receptors in the *Dictyostelium* cellular membrane detect the presence of folic acid in the surrounding. Intracellularly this results in dissociation of Gα and Gβγ subunits, activation of target enzymes and eventually in chemotaxis of the amoebae in the direction of the increasing folic acid concentration. The sensitivity to folic acid is lost upon starvation and chemotaxis is subsequently controlled by cAMP. This shift in chemotactic attraction is accompanied by a rise in the level of cAR1 mRNA, leading to an increasing amount of molecules of the cell surface cAMP receptor.

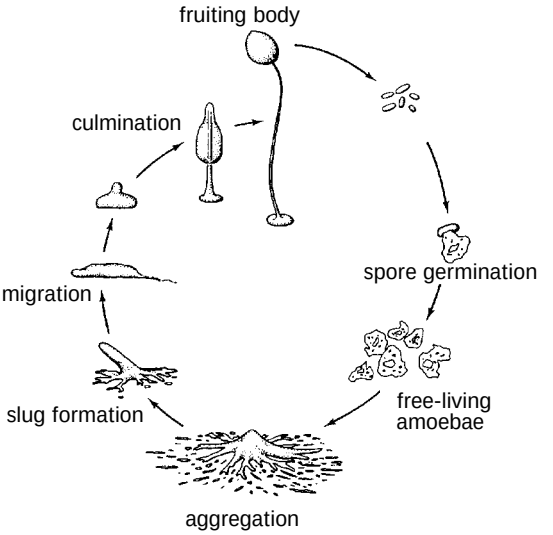

Figure 5. *Lifecycle of Dictyostelium discoideum*
Under favourable conditions *Dictyostelium* spores germinate and start feeding and multiplying. Consumption of the foodsource leads to aggregation of the free living amoebae into a multicellular slug. The slug falls over and migrates towards more promising parts of the surroundings. Finally the fruiting body with its stalk cells and spore cells develop from where the spore are spread in the area.

COMPONENTS OF THE DICTYOSTELIUM SIGNAL TRANSDUCTION MACHINERY

Cloning of genes involved in signal transduction

The cAMP receptor belongs to the group of seven transmembrane receptors. In *Dictyostelium* four genes coding for cAMP receptors were identified thus far (fig. 6) (Klein *et al*., 1988; Saxe *et al*., 1991a,b; Saxe *et al.*, 1993). Messenger RNA of the cAMP receptor cAR1 appears at low levels in vegetative cells and increases a few hours after starvation. After the aggregation stage the mRNA level of cAR3 rises, in the fifth hour of development, followed by cAR2 and cAR4.

Dictyostelium discoideum the search for In genes that code for the subunits of the G-protein has thus far revealed eight different genes for the Gα subunit and one gene coding for Gβ; genes for the Gγ-subunit have not been discovered yet (Wu & Devreotes, 1991; Lilly *et al*., 1993). Experiments with Gβ disruptants indicate that *Dictyostelium* only has one gene coding for the β-subunit, that is continuously expressed during development (Wu *et al.*, 1995).

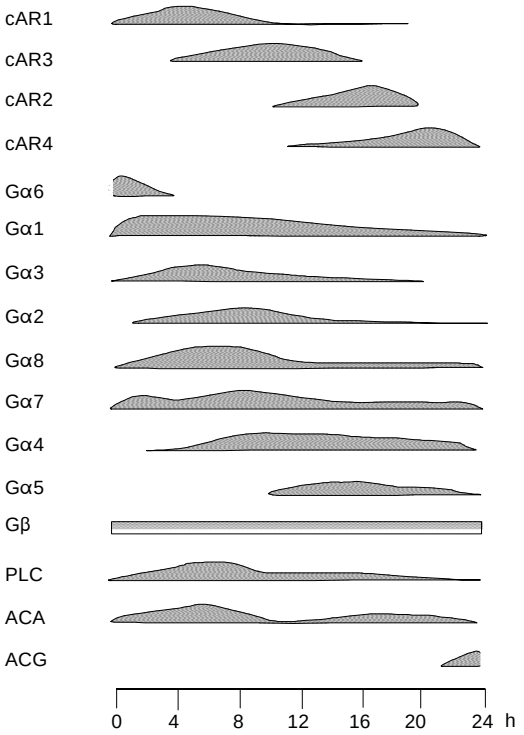

Figure 6. *Time schedule representing expression of presently cloned genes involved with signal transduction in* Dictyostelium discoideum
cAR1-4: cAMP receptors; Gα1-8: α subunits of G-protein; Gβ: β-subunit of G-protein; PLC: phospholipase C; ACA: adenylyl cyclase A; ACG: adenylyl cyclase G.

The cAMP response and relay

Cyclic AMP serves universally as a second messenger. It activates several target molecules- primarily cAMP dependent protein kinases- to control such diverse phenomena as metabolism, gene transcription and memory. In *Dictyostelium*, cAMP functions both as a first messenger and as a second messenger. Excitation of the cell surface cAMP receptor results in stimulation of the adenylyl cyclase activity, which gives a rise in the cellular cAMP content, reaching its maximum one to two minutes later. Intracellular cAMP levels then decrease slowly, to reach rest values five to ten minutes after excitation. Extracellularly an increase of cAMP is also observed, due to secretion of the produced cAMP, an event called relay (fig. 7) (Devreotes & Steck, 1979; Van Haastert, 1984, 1987a).

Two adenylyl cyclases have been identified in *Dictyostelium*, one of which, ACA, is expressed maximally in early development. The second adenylyl cyclase, ACG, is present during the germination stage in spores. Clearly ACA is the adenylyl cyclase that is responsible for all developmental processes, except spore germination, in which cAMP is involved.

Adenylyl cyclase A can be activated by both cAR1 and cAR3 stimulation, coupled to G-protein $G\alpha_2\beta\gamma$ (Pupillo *et al.*, 1992; Insall *et al.*, 1994a) which dissociates into $G\alpha_2$ and Gβγ. ACA is not activated in mutants lacking cAR1, Gα2 or Gβ (*car1*$^-$, *gα2*$^-$ and *gβ*$^-$). In lysates, addition of GTPγS results in activation of ACA in *car1*$^-$ and *gα2*$^-$ but not in *gβ*$^-$cells. Another essential protein for the activation of ACA is the cytosolic factor CRAC. In *synag7* mutants, which lack CRAC, ACA fails to be activated (Theibert & Devreotes, 1986). Lilly and Devreotes (1995) showed that chemoattractants promote the translocation of CRAC from the cytosol to the membrane; translocation was still observed in mutants lacking ACA, but was absent in *gβ*$^-$cells. This observation led to the proposition of a model in which the released Gβγ subunit recruits CRAC to the membrane in order to establish a membrane bound complex that subsequently activates ACA (Wu *et al.*, 1995; Lilly & Devreotes, 1995). Finally, also involved in the activation of ACA is a MAP kinase, but the underlying mechanism is still unclear (Segall *et al.*, 1995).

The cGMP signalling response

The second messenger cGMP acts as an intracellular signalling molecule, the target being protein kinases, cyclic nucleotide phosphodiesterases, myosin and possibly some other proteins. In *Dictyostelium* stimulation of guanylyl cyclase was observed by folic acid and cAMP (Mato *et al.*, 1977a), and osmotic stress (Kuwayama *et al.,* 1996). Intracellular cGMP regulates the assembly and disassembly of myosin filaments by inducing the phosphoryla-tion of three threonine residues (Liu *et al.*, 1993).

Guanylyl cyclase shows a fast response upon stimulation with cAMP in aggregation competent cells: within one second after ligand binding to the cAMP receptor the basal level of cGMP rises. The cGMP concentration peaks after 10 - 15 seconds and decreases to return to basal values after 30-45 seconds (fig. 8). The recovery of the cGMP response is a cooperation between many components in the cell, including receptor adaptation, Ca^{2+} concentration, degradation of cGMP by cGMP-specific phospho-diesterase (cGMP-PDE) (Van Haastert *et al.*, 1983; Valkema & Van Haastert, 1994). Like guanylyl cyclases in the photoreceptor outer segment, *Dictyostelium* guanylyl cyclase activity increases when the Ca^{2+} level is lowered (fig. 7). The specific degradation of cGMP by a phosphodiesterase that becomes activated by cGMP is an important regulator of the cGMP response. *Stm*F mutants which lack the cGMP-PDE show an aberrant cGMP response upon receptor excitation, with a large increase of cGMP, and the return to basal levels taking several minutes (Ross & Newell, 1981; Van Haastert *et al.*, 1982).

Kuwayama *et al*. (1993) described a set of mutants that neither show chemotaxis towards cAMP or folic acid, nor development after starvation. These mutants appeared to have an abnormal cGMP synthesis. Mutant KI-8 has a defect in basal cGMP production, with intracellular cGMP concentration below detection levels. Mutant KI-10 has basal levels of cGMP, but no guanylyl cyclase activation is detectable after stimulation with cAMP or folic acid. The defect in mutant KI-8 lies probably in a totally inactive or even absent guanylyl cyclase, whereas KI-10 can have both a mutation in guanylyl cyclase or a mutation in a protein that interacts between guanylyl cyclase and the receptor-G-protein complex. Recently evidence was found that a cGMP-binding protein is involved in a feedback loop of regulating guanylyl cyclase activity in *Dictyostelium* (Kuwayama & Van Haastert, 1996).

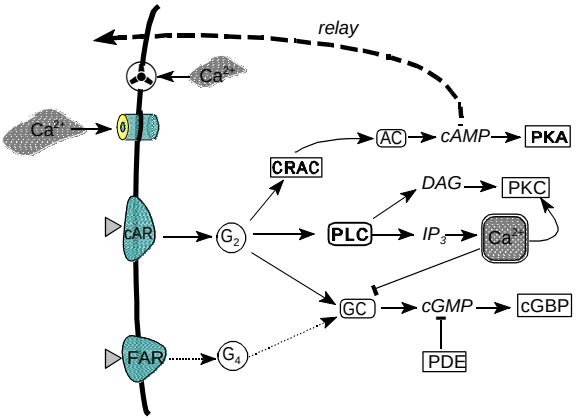

Figure 7. *General scheme of signal transduction in Dictyostelium.*
cAR: cAMP receptor; FAR: Folic acid receptor; G: G-protein; CRAC: cytosolic regulator of adenylyl cyclase; AC: adenylyl cyclase; PLC: phospholipase C; GC: guanylyl cyclase; PDE: phosphodiesterase; PKA: cAMP dependent protein kinase; PKC: protein kinase C; cGBP: cGMP binding protein

The cAMP mediated $InsP_3$ response

Two important second messengers,

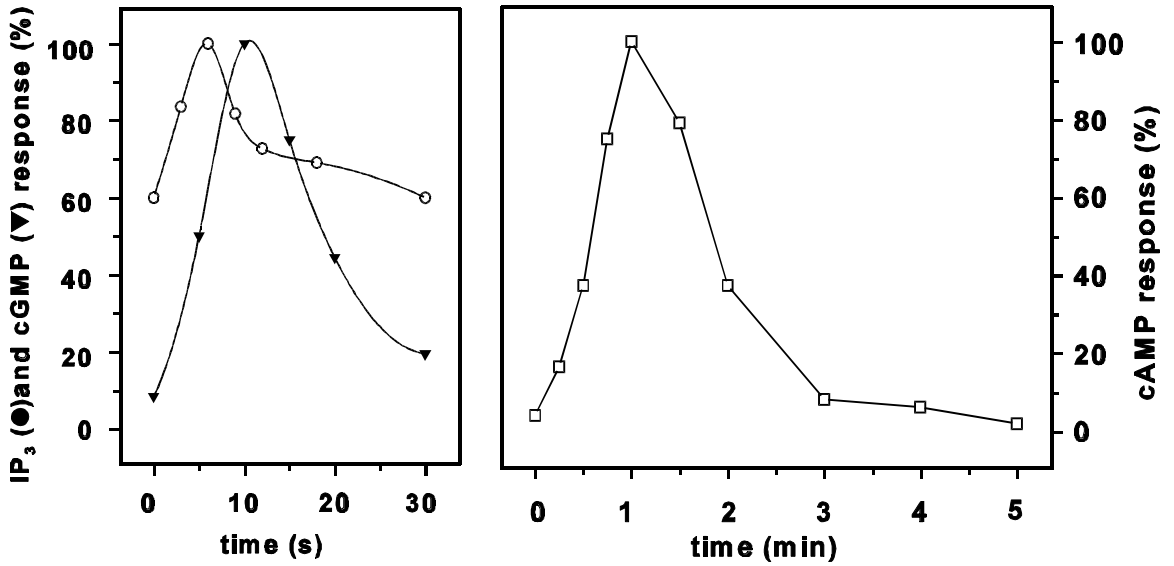

Figure 8. *The three most important cAMP induced second messenger responses in Dictyostelium discoideum*.
Left panel: IP_3 response (○) and cGMP response (▼). Right panel: cAMP response (□)

inositol-1,4,5 trisphosphate ($InsP_3$) and diacylglycerol (DAG), are released when phosphatidylinositol-4,5-bisphosphate ($PtdIns-P_2$) is hydrolysed by phospholipase C. PLC activity is stimulated by the cAMP receptor in developing cells. When cells are exposed to cAMP, the cellular $InsP_3$ level rises to reach peak values after six seconds. The $InsP_3$ concentration then gradually decreases again to reach basal levels 30 seconds later. $InsP_3$ is known to play a role in release of Ca^{2+} from non-mitochondrial stores, whereas DAG is involved in the activation of PKC (Europe-Finner & Newell, 1986a; Kishimoto *et al.*, 1980) (fig. 7).

In *Dictyostelium*, a *plc*$^-$ mutant was constructed by gene disruption (Drayer & Van Haastert, 1992; Drayer *et al.*, 1994). Surprisingly, the development of this cell line appeared to be normal, and biochemical investigation showed that $InsP_3$ level, despite the lack of PLC-activity, is not significantly different from wild-type cells. Recently, Van Dijken *et al*. (1995) showed that in *Dictyostelium* an alternative route exists by which $InsP_3$ is a product of degradation of higher inositols phosphates, which may explain why PLC is not essential for viability and development in *Dictyostelium*.

Content of this thesis

In this thesis a number of studies on different aspects of signal transduction in *Dictyostelium discoideum* are presented. Chapter 2 concentrates on the role of phosphorylatable serines in the C-terminus of the cAMP receptor cAR1 in the adaptation and deadaptation of guanylyl and adenylyl cyclase responses.

The following chapter is a study on the characteristics of the germination stage specific adenylyl cyclase ACG. This cyclase was expressed in a mutant strain which lacks the aggregation stage specific adenylyl cyclase ACA. Stimulation of the mutant cell line with cAMP caused activation of ACG, in analogy with ACA stimulation. In contrast with ACA, stimulation of ACG enzyme activity with cAMP does not require G-protein interaction. Also a distinguishing feature of ACG is the activation by stimulation of the folic acid receptors in vegetative cells.

Chapter 4 describes three studies concerning guanylyl cyclase, the cGMP synthesizing enzyme. In the first report we describe that *Dictyostelium* guanylyl cyclase is inhibited by the presence of submicromolar concentrations of Ca^{2+}, in contrast to the related enzymes in many other organisms. In the second paper the cGMP response was analysed in a computer model. In the model we tested our knowledge of the kinetic properties of guanylyl cyclase and cGMP-phospho-diesterase as measured in several experiments and we incorporated four adaptation models of the cAMP receptor. Finally the chapter ends with an overview of the strategies used in the search for the GC gene in *Dictyostelium*.

The final chapter provides a summary of the preceding text and a short discussion of the results compared with recent publications.

Differential role of surface cAMP-receptor phosphorylation in adaptation and deadaptation of adenylyl cyclase and guanylyl cyclase in *Dictyostelium discoideum*

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Chapter 2

ABSTRACT

In *Dictyostelium discoideum,* extracellular cAMP activates adenylyl and guanylyl cyclase, which become adapted within a few minutes after prolonged receptor stimulation. The cell surface cAMP receptor cAR1 contains four serine clusters in the cytosolic C-terminus, two of which are involved in the ligand induced phosphorylation. Phosphorylation of the receptor serines is thought to have a major contribution to the process of adaptation and deadaptation. We investigated the role of receptor phosphorylation in a cell line that expresses the mutant receptor form CM1234, which lacks all C-terminal serines. The kinetics of the cAMP-mediated guanylyl cyclase response, as well as its adaptation and deadaptation, have the same characteristics in the mutant cell line as in wild type *Dictyostelium* cells. The kinetics of the adenylyl cyclase response after stimulation of the mutant receptor, and its adaptation, resemble the reactions in wild type cells. However, within 10 s after removal of the stimulus, mutant cells are responsive to restimulation, whereas wild-type cells regain responsiveness more slowly, with a half life of 3-4 minutes. Our observations suggest that the phosphorylation sites in the receptor are not required for the activation and adaptation of adenylyl and guanylyl cyclase or deadaptation of guanylyl cyclase, but specifically affect deadaptation of adenylyl cyclase.

INTRODUCTION

The study of signal transduction explores intracellular and intercellular communication. In eukaryotic cells, this involves the interaction of first messengers (hormones) with their receptor, which are found inside the cell or at the cell surface in case of the transmembrane receptors. In the lower eukaryote *Dictyostelium discoideum*, the best understood cell surface receptor is the cAMP receptor that controls the processes of chemotaxis, aggregate formation, slug migration and differentiation.

The major part of the *Dictyostelium* life cycle concerns the vegetative stage, when the unicellular amoebae live in the soil and feed on bacteria. Upon depletion of the food source, cells organize and form aggregates of up to 100,000 cells, out of which in the final stage a fruiting body is formed. The unicellular amoebae eventually differentiate into two cell types: viable spore cells which are found in the spore head on top of dead stalk cells. During the vegetative stage, *Dictyostelium* detects and moves towards its bacterial food source by means of chemotaxis. At this stage chemotaxis is induced by folic acid, a secretion product of bacteria that is sensed by cell surface folic acid receptors. Food depletion induces cAMP synthesis. Cyclic AMP is secreted to the cell exterior, where it is detected by cell surface cAMP receptors on neighbouring cells. These cells accordingly become involved in the chemotactic process that leads to aggregate formation and development into a fruiting body. Extracellular cAMP also induces gene expression: in the first instance the aggregative genes (encoding for e.g., cAMP receptor cAR1, cAMP-phosphodiesterase and G-protein subunit Gα2 (Klein *et al.,* 1988; Lacombe *et al.*, 1986; Kumagai *et al.,* 1989) and later on the prespore and prestalk genes (Williams, 1988).

The *Dictyostelium* genome revealed four genes that code for cAMP receptors, cAR1 to 4 (Klein *et al.*, 1988; Saxe *et al.*,

1991). During aggregation, cAR1 is maximally expressed, followed by cAR3, while cAR2 and cAR4 messengers appear in the multicellular stage of development. Receptor stimulation evokes a signal that is passed on, via a hetero-trimeric G-protein, to effector enzymes that include adenylyl cyclase, guanylyl cyclase and phospholipase C. Receptor stimulation also leads to opening of Ca^{2+} channels to let Ca^{2+} enter the cell, an event that occurs independently of G-proteins (Milne *et al.*, 1995). In reaction to receptor stimulation, the effector enzymes phospholipase C, guanylyl cyclase and adenylyl cyclase produce an increase in second messenger levels (IP3, cGMP and cAMP respectively). Second messenger concentrations rise to a maximum at different time points and decrease again to basal levels (Roos & Gerisch, 1976; Mato *et al.*, 1977; Devreotes & Steck, 1979; Van Haastert *et al.*, 1989).

Prolonged exposure of the cell to a cAMP stimulus of constant concentration first induces responses and subsequently makes the cell insensitive to further stimulation, a mechanism called adaptation. Removal of the stimulus reverses the process of adaptation, the cell recovers responsiveness in a few minutes (Dinauer *et al.*, 1980; Van Haastert & Van der Heijden, 1983). In earlier investigations it has been noticed that adaptation of the cAMP mediated adenylyl cyclase response shows the same kinetics as the phosphorylation of the cAMP receptor. The cAMP receptor cAR1 is found in two interconvertible iso-forms: the R-form and the D-form. The D-form shows a fivefold increased phosphorylation level; its occurrence is closely related to the state of adaptation of the cell (Klein *et al.*, 1985; Vaughan & Devreotes, 1988). The receptor contains four clusters of serine residues in the C-terminal tail. Mutations in these clusters have pointed out the regions of basal and ligand stimulated phosphorylation (Hereld *et al.*, 1994).

We tested the hypothesis that receptor phosphorylation provides the mechanism of adaptation of guanylyl and adenylyl cyclase using a mutant cell line, where cAR1 is replaced by a modified cAMP receptor CM1234 in which all four serine clusters are removed. The mutant receptor cannot be phosphorylated anymore upon cAMP stimulation. We discovered that the role of receptor phosphorylation is not identical in the guanylyl and the adenylyl cyclase response.

MATERIALS & METHODS

Materials

[2,8-^{3}H]cAMP (50 Ci/mmol) and cGMP radio immunoassay kit were obtained from Amersham; 2′-deoxy-cAMP was from Sigma. DTT and cAMP were from Boehringer Mannheim; KH_2PO_4, Na_2HPO_4, $HClO_3$ and $KHCO_3$ were from Merck.

Cells and growth conditions

CM1234/JB4 cells were made by electroporation of JB4 cells (which are *car1*$^-$ by gene disruption) with CM1234 plasmid (Hereld *et al.*, 1994). Cells were grown in shaking culture at 22°C in HG5 medium (Watts & Ashworth, 1970), CM1234/JB4 cells were grown in the presence of 10 µg/ml G418. Cells were harvested at late-logarithmic phase and starved on a rotary shaker in 10 mM KH_2PO_4/Na_2HPO_4 pH 6.5 (phosphate buffer, PB) at a cell density of 10^7 ml^1. Cells were starved for 5 hours and were pulsed every 5 minutes with 0.1 µM cAMP

from t=2 to t=5 h. Subsequently cells were washed twice, resuspended in PB at a final density of 5.10^7.ml^{-1}, and aerated for at least 15 minutes at 22°C.

Stimulation of cells

Cells at a density of $5x10^7$ per ml were mixed with a one-tenth volume stimulus (final concentrations indicated in the legends of the figures) and incubated at 22°C. At given time points 50 µl samples were withdrawn in which the reaction was stopped by lysing the cells by adding 50 µl 3.5% (v/v) $HClO_3$. The lysate was neutralized with 25 µl $KHCO_3$ (50% saturated at 20°C). Cyclic AMP contents were measured by isotope dilution, using the regulatory subunit of cAMP-dependent protein kinase type I. As a cAMP stimulus would interfere with the assay of cAMP produced by adenylyl cyclase, the stimulus 2′-deoxyadenosine 3′,5′-phosphate (dcAMP) was used; this cAMP analogue binds effectively to surface cAMP receptors, but very poorly to the assay binding protein cAMP-dependent protein kinase. The cGMP content was measured by means of a radio-immunoassay.

Deadaptation of stimulated adenylyl cyclase

Cells were stimulated with dcAMP (10 µM final concentration) and samples were withdrawn for the next five minutes. At t=4 min the cells were chilled on ice and spun down at t=5 min for 15 s at 300 g. The supernatant was removed and the cells were resuspended in one volume fresh PB (22°C). At different time points after resuspending (between 10 s and 4 min) cells were restimulated by addition of 5 µl stimulus to 45 µl of cell suspension. Reactions were stopped after 2 min by adding 50 µl $HClO_3$. After neutralising with 50% saturated $KHCO_3$, the total cAMP content was measured.

RESULTS

Stimulation of CM1234 activates guanylyl and adenylyl cyclase

To determine the contribution of the phosphorylatable serine residues in the cytosolic C-terminus of the cAMP receptor to the transient activation of these cyclases, we measured the levels of cGMP and cAMP after addition of a dcAMP stimulus to CM1234/JB4 cells. In this cell line, which has a deletion of the cAR1 gene, a mutant cAR1 receptor is expressed that lacks all C-terminal serine residues (Hereld *et al.*, 1994).

Stimulation of CM1234/JB4 cells with dcAMP leads to a transient accumulation of cGMP (fig. 1A). Ten seconds after receptor stimulation the intracellular levels of cGMP have increased tenfold, and basal levels are regained after 45 seconds. This activation of guanylyl cyclase resembles a typical guanylyl cyclase response of wild type cells (Van Haastert & Van der Heijden, 1983). Excitation of the mutant receptor also induces cAMP production (fig. 1B). The concentration of cAMP reaches its maximum after 2 minutes, and has returned to basal amounts within 10 minutes after stimulation. The produced cAMP is subsequently secreted (data not shown). The magnitude and kinetics of

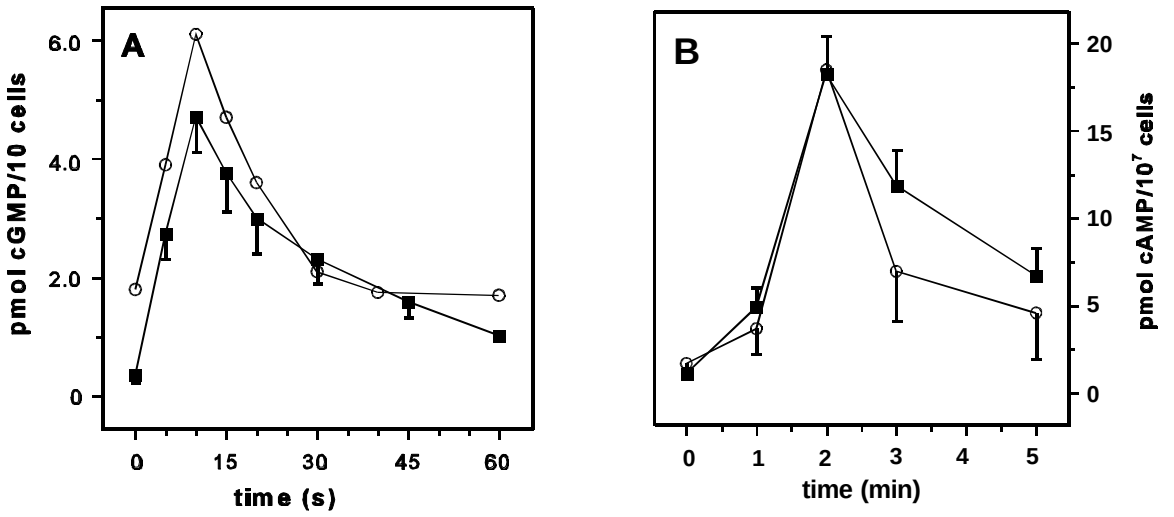


Figure 1 A. *Activation of guanylyl cyclase by dcAMP stimulated CM1234 receptor.*
Cells were stimulated at t=0 s. At indicated time points samples were taken and reactions were stopped by addition of one volume 3.5% percloric acid. After neutralisation with $KHCO_3$ cGMP levels were measured. (■), cGMP accumulation in CM1234/JB4 cells stimulated with 5µM dcAMP + 5 mM DTT, means ± S.E.M. of 5 experiments in triplicate; (○), cGMP response in wild-type AX3 cells, stimulated with 10 nM cAMP.

B. *Activation of adenylyl cyclase by dcAMP stimulated CM1234 receptor.*
Cells were stimulated and treated as described above. After neutralisation cAMP levels were measured. (■), cAMP accumulation in CM1234/JB4 cells stimulated with 5µM dcAMP + 5 mM DTT, means ± S.E.M. of 10 experiments in triplicate; (○), control: wild-type AX3 cells stimulated with 10 µM dcAMP, means ± S.E.M. of 2 experiments in triplicate.

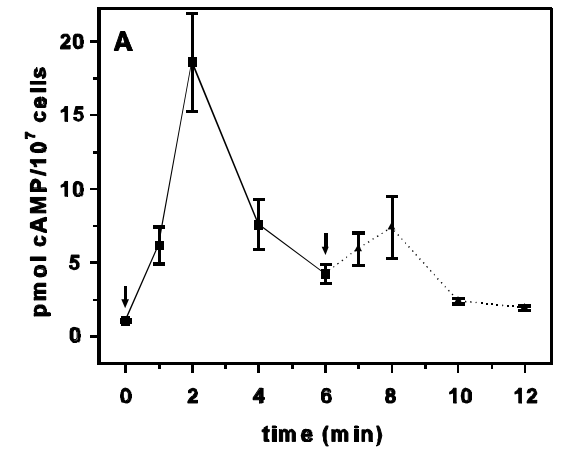
the observed adenylyl cyclase response in CM1234/JB4 cells are in close agreement with the response reported for wild type cells (Devreotes & Steck, 1979). Both the adenylyl cyclase response and the guanylyl cyclase response have a transient character, which suggests that, despite the removal of all phosphoryl-atable serines in the cAMP receptor, these receptor-mediated responses desensitize.

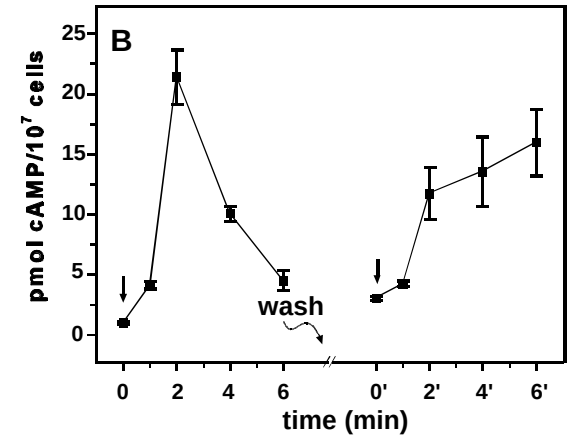
Adaptation of the adenylyl cyclase response

To determine whether activation of adenylyl cyclase is subject to adaptation, we measured the cAMP production of CM1234/JB4 cells after two successive stimuli applied at a six minute interval, without washing the cells in between. The first stimulus induces a full response; we observed that the second stimulus induced only partial excitation of adenylyl cyclase activity (fig. 2A). Two minutes after the second stimulation cAMP production peaks at a level that is only 17% of the response induced by the first stimulus. From this we conclude that the first stimulus has induced nearly complete adaptation of the cAMP receptor mediated adenylyl cyclase response in CM1234/JB4 cells, in a way similar to that in wild-type cells.

Deadaptation of the adenylyl cyclase response

Adapted wild-type cells washed to remove the stimulus, slowly regain





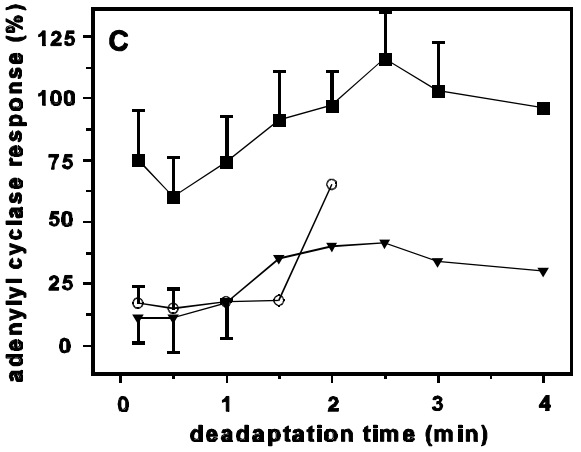


Figure 2 A. *Adaptation of the cAMP receptor mediated adenylyl cyclase response.*
Cells were stimulated with 5 µM dcAMP +10 mM DTT at t=0 min. Samples were taken at indicated time points. A second stimulus was given at t=6 min. and additional samples were obtained. Total cAMP levels were measured. Data represent means ± S.E.M. of three experiments in triplicate. The response to the second stimulus is only 17% if compared to the first response, indicating that cells were adapted to the first stimulus.

B. *Deadaptation of the cAMP receptor mediated adenylyl cyclase response.*
Cells were stimulated with 10 µM dcAMP and samples were taken at indicated time points. At t=4 min cells were chilled in ice water, at t=5 min. the cells were spun down for 15 s at 300 g and the supernatant was removed. The cells were resuspended in the original volume PB at room temperature, and the second stimulus (10 µM dcAMP) was applied, indicated by t=0'. Samples were withdrawn at indicated time points and reactions were stopped by adding 50 μl $HClO_3$. After neutralisation with 50% saturated $KHCO_3$ total cAMP levels were measured, data represent means ± S.E.M of three experiments in triplicate. The response to the second stimulus is 63% compared to the first response, indicating that the cells were deadapted during the washing step.

C. *Level of deadaptation of washed cells stimulated at various time points after resuspending*.
Starved cells (CM1234/JB4 and wild-type AX3) were stimulated with 10 µM dcAMP and the next 5 min cell samples were withdrawn and lysed. Cells were chilled to 0°C at t=4 min, spun down at t=5 min (15 s, 325 g, 0°C). The first stimulus was aspirated and cells were resuspended in fresh PB, 22°C at t=6 min. Cells received a second 10 µM dcAMP stimulus at different time points after resuspension and were lysed 2 min after the second stimulus. Control CM1234/JB4 cells were not cooled and washed, but were stimulated in a similar way. At the X-axis t=0 s represents the point of resuspending the cells, the next time points represent the time elapsed before cell samples received the second stimulus (deadaptation time). Cyclic AMP levels represent the amount of total cAMP measured 2 min after the second stimulus. (■), CM1234/JB4 cells, means ± S.E.M. of 10 experiments in triplicate; (▼), CM1234/JB4 cells control, not washed, means ± S.E.M. of 7 experiments in triplicate; (○), AX3 cells, means ± S.E.M. of 3 experiments in triplicate. Points without an error bar were obtained from single experiments.

responsiveness (deadapt). In AX3 cells deadaptation of adenylyl cyclase after cAMP receptor stimulation occurs with a half-life of 3-4 minutes; however, at 0°C deadaptation is absent (Dinauer *et al.,* 1980; Van Haastert, 1987). We analysed the deadaptation rate of the adenylyl cyclase response in CM1234/JB4 cells after inducing adaptation with a saturating dcAMP stimulus. Cells were stimulated (t=0 min) with a saturating concentration of dcAMP; after 4 minutes cells were placed on ice and spun down to remove the buffer with stimulus. At t=6 min cells were resuspended in fresh buffer at 22°C and at increasing time intervals cell batches were mixed with a second dcAMP stimulus. The cells were allowed to react to the second stimulus for exactly two minutes. We observed that restimulation at 10 seconds after removal of the first stimulus induced a large cAMP response that reaches a magnitude of 75% of the first cAMP accumulation (fig. 2B). Allowing the cells a longer interval for deadaptation leads to a full recovery of responsiveness within 90 s (fig. 2C). When CM1234/JB4 cells are not washed after the first stimulus, the level of the second cAMP peak only reaches 11% of the first response. In control experiments with wild-type AX3 cells, restimulated within one minute after removal of the first stimulus, the response to the second stimulus reaches a level of 17% of the first cAMP peak. When the second stimulus is applied later after removal of the first stimulus, wild-type cells show a slow recovery of responsiveness.

Thus, the observed deadaptation of CM1234/JB4 cells differs from the deadaptation of the cAMP response in wild-type cells. It appears that cells carrying the mutant receptor remain adapted as long as the stimulus is present, but deadapt within 10 s after removal of the stimulus. In contrast, wild-type cells deadapt with a half life of 3-4 minutes. From these results we conclude that the mutations of the serines in the cytosolic loop of the cAMP receptor have a major influence on the deadaptation process of the cAMP receptor mediated stimulation of adenylyl cyclase activity.

Adaptation of the guanylyl cyclase response

To find out wether preventing phosphorylation of the cAMP receptor affects the adaptation of the guanylyl cyclase response, we determined the cGMP response in cells that were prestimulated with cAMP. The cGMP synthesis after two 100 nM cAMP stimuli is shown in figure 3A. It appears that 50 s after stimulation of guanylyl cyclase activity by a saturating cAMP dose the CM1234/JB4 cells do not effectively react to a new stimulus. The second stimulus induces only a small increase of cGMP levels, with a peak that is only 17 % of the first cGMP accumulation. The magnitude of

Table 1. *Adaptation of cGMP response in CM1234/JB4 at different stimulus concentrations.*
Starved CM1234/JB4 cells were stimulated with cAMP at t=0 s and t= 50 s. Cell samples were withdrawn at 0, 12, 50 and 62 s. Results are means ± S.E.M. from 3 experiments (n=3, 4).

Stimulus (nM)		cGMP response to 2nd stimulus
1rst	2nd	(% of 100 nM cAMP)
-	100	100% (p. def.)
-	10	68 ± 14
100	100	-6 ± 3
10	10	-4 ± 3
10	100	40 ± 22

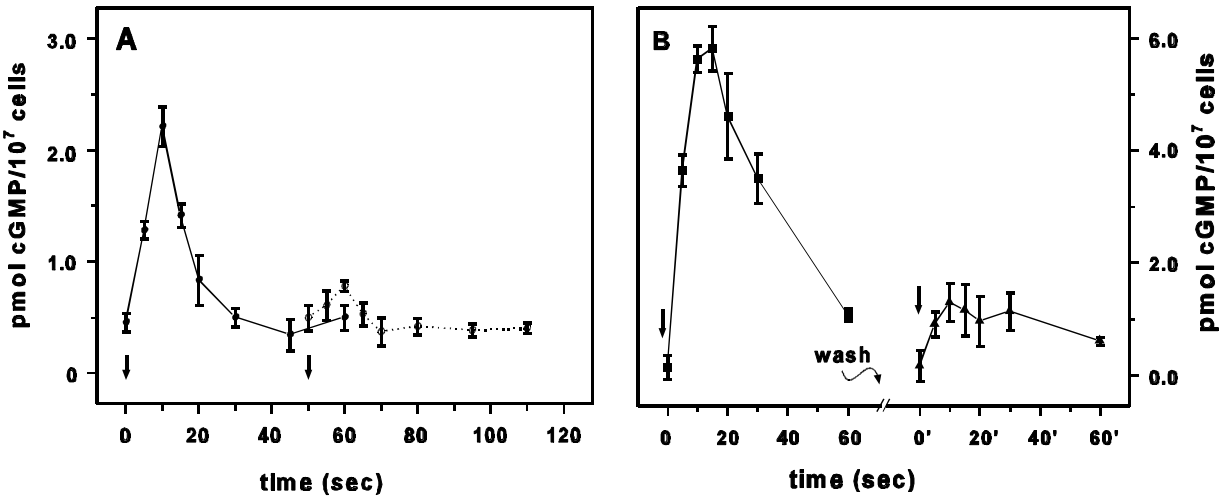


Figure 3 A. *Adaptation of CM1234 receptor stimulated guanylyl cyclase response*.
Cells were stimulated with 100 nM cAMP and samples were taken at indicated time points. At t=50 s part of the cells received a second stimulus of 100 nM cAMP. Data represent means ± S.E.M. of 2 experiments in triplicate.

B. *Deadaptation of CM1234 receptor stimulated guanylyl cyclase response.*
Cells were stimulated with 5 µM dcAMP + 10 mM DTT at t=0 s. Samples were taken at indicated time points. From t=5 min cells were treated as described in figure 2B. At t=10 min the cells were resuspended in one volume of PB (room temperature) and a second stimulus was applied. Data represent means ± S.E.M. of 3 experiments in triplicate. Arrows indicate time point of stimulus application (straight) and wash step (curved). The results of figures 2b and 3B were obtained from the same batches of cells.

the response to the second stimulus is similar to that in wild-type cells.

Typically, adapted cells do not respond to a second stimulus with a concentration lower than, or similar to the first stimulus. When the concentration of the second stimulus is higher than the first stimulus, cells do respond to the second stimulus. This second response is additive: the sum of the two responses equals the magnitude of a single response elicited by a stimulus of the highest concentration (Van Haastert, 1983; Dinauer *et al.*, 1980). To test if the observed desensitization of the guanylyl cyclase response also obeys additivity, we measured the cGMP production in CM1234/JB4 cells at 12 s after stimulation with different pairs of cAMP concentra-tions (table 1). It appears that 50 s after a first stimulus (10 or 100 nM), the cells are insensitive to a second stimulation of equal concentration. However, cells stimulated with 10 nM cAMP and restimulated with 100 nM cAMP produce two cGMP waves, with a total concen-tration equal to a cGMP accumulation after a single 100 nM cAMP stimulus. These results indicate that CM1234/JB4 cells show adaptation of the cGMP response, indistinguishable from adaptation in wild-type cells.

Deadaptation of the guanylyl cyclase *response*

To determine whether adaptation of guanylyl cyclase activity is erased upon removal of the stimulus, analogous to the rapid deadaptation of adenylyl cyclase, we measured the cGMP response in cells washed with PB after a first stimulus. We noticed that after removal of the first stimulus, application of a second cAMP stimulus to CM1234/JB4 cells does not

induce the activation of guanylyl cyclase (fig. 3B). These results indicate that guanylyl cyclase is not deadapted within 10 s after washing away the stimulus, in contrast to what was found for adenylyl cyclase. It is important to mention that these results on deadaptation of guanylyl cyclase (fig. 3B) were obtained using the same cell batches as were used for the determination of deadaptation of adenylyl cyclase (fig. 2B). It thus appears that both the adaptation and deadaptation processes of guanylyl cyclase in CM1234/JB4 cells are essentially not different from those in wild-type cells.

DISCUSSION

Transmembrane receptors maintain the communication between the extra-cellular compartment and the cellular interior. The *Dictyostelium* cAMP receptor is a member of the family of seven transmembrane receptors that transduce extracellular signals to intracellular effectors via G-proteins (reviewed in Dohlman *et al.,* 1991). Many responses mediated by receptors of this type show desensitization after prolonged stimulation with ligand. Upon generating an initial response, the transduction system becomes refractory to further stimulation. The presence of ligand for still longer periods leads to reduction in the number of receptor molecules. In the β2 adrenergic receptor short-term desensitization and the loss of G-protein coupling are associated with receptor phosphorylation (Strader *et al*., 1987; Bouvier *et al.*, 1988; Pitcher *et al*., 1992; Eason *et al*., 1995; Freedman *et al*., 1995).

In *Dictyostelium,* the cAMP receptor becomes excited by extracellular cAMP, leading to the activation of various enzymes. Guanylyl cyclase and adenylyl cyclase show peak activities at 10 s and 120 s after cAMP addition, respectively. Both responses are subject to adaptation: continued stimulation of the cAMP receptor leads to termination of the increase in enzyme activity (Mato *et al.*, 1977; Devreotes & Steck, 1979; Van Haastert *et al.*, 1989). cAR1 possesses C-terminal serines that become phosphorylated upon excitation by ligand binding (Klein *et al.*, 1985; Klein *et al.,* 1988). These serines are located in four clusters, of which the first two clusters are involved in the ligand induced phosphorylation (Hereld *et al.*, 1994). The kinetics and dose dependency of receptor phosphorylation coincide with the kinetics and dose dependency of adaptation of the receptor mediated activation of adenylyl cyclase. In analogy with the putative role of mammalian receptor phosphorylation it was proposed that phosphorylation of cAR1 may be the molecular mechanism of adaptation in *Dictyostelium*. Mutation of all C-terminal serines yields a receptor called CM1234 that displays no phosphorylation upon ligand binding (Hereld *et al.,* 1994). Expression of this receptor in a *Dictyostelium* mutant that does not express endogenous cAR1 provides the unique opportunity to investigate the role of receptor phosphorylation in signal transduction and desensitization.

We investigated the effect of the mutation of the C-terminal serine-residues in the cAMP receptor CM1234 on the activation and adaptation of guanylyl and adenylyl cyclase. We observed that the kinetics and magnitude of the accumulation of both cAMP and cGMP levels are equal to what was observed in wild-type cells (Devreotes & Steck, 1979; Mato *et al.*, 1977), indicating that the excitation of both cyclases is not altered by the removal or mutation of

phosphorylatable serine residues from the receptor. The observed recovery of basal values of cAMP and cGMP levels after receptor stimulation of CM1234 cells suggests that the activation of the enzymes was subject to adaptation. When CM1234 cells were stimulated twice at a relatively short interval, we observed that cells did not respond to the second stimulus with an increase of either cAMP or cGMP levels. We therefore conclude that the cAMP stimulus induces adaptation of both adenylyl cyclase and guanylyl cyclase in a mutant carrying a cAMP receptor without phosphorylatable serines in the C-terminal tail. Upon removal of the stimulus, wild-type cells slowly regain responsiveness to cAMP with a half-life of recovery of 2-4 minutes for both adenylyl cyclase and guanylyl cyclase (Dinauer *et al.*, 1980; Van Haastert, 1987). We observed essentially the same kinetics for desensitization of guanylyl cyclase in CM1234 cells as in wild-type cells, leading to the conclusion that activation, adaptation and deadaptation of receptor-stimulated guanylyl cyclase are all unaltered in CM1234 cells. In contrast, deadaptation of adenylyl cyclase was very fast in CM1234 cells: within 10 s after removal of cAMP, readdition of the stimulus induced a nearly full activation of adenylyl cyclase.

It thus appears that deadaptation of adenylyl cyclase and guanylyl cyclase are different in CM1234 cells. This conclusion expands previous observations that also the mechanism of adaptation is different for these cyclases. The kinetics of adaptation of adenylyl cyclase is relatively temperature independent, whereas adaptation of guanylyl cyclase is essentially absent at 0°C; deadaptation shows the opposite

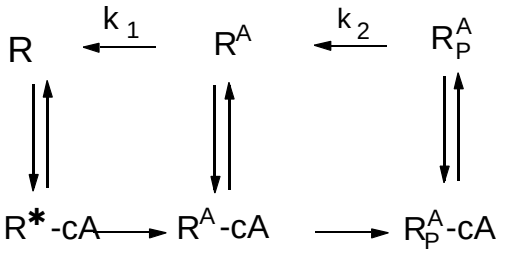


Figure 4. *Scheme of receptor model of the cAMP response.*
R: unoccupied receptor in the ground state. R*-cA: occupied active receptor, R^A-cA: adapted inactive form, occupied by ligand. R^A: ligand free form of the inactive adapted receptor. R^A_P-cA: adapted, phosphorylated receptor isoform, ligand occupied and inactive. R^A_P: unoccupied form of the phosphorylated adapted receptor. k_1: rate constant of decay of adapted state R^A to R, $>6.9\times10^{-2}$ s^{-1}, k_2 : rate constant of dephosphorylation from R^A_P to R^A, 3.6×10^{-3} s^{-1}. The model is explained in the discussion.

temperature dependency at 0°C: it is absent for adenylyl cyclase and slow for guanylyl cyclase (Van Haastert, 1987). The present observation that in CM1234 cells deadaptation of adenylyl cyclase is extremely fast, whereas deadaptation of guanylyl cyclase is essentially normal, confirms the conclusion that the molecular mechanisms of adaptation of adenylyl cyclase and guanylyl cyclase are different.

Our observations implicate that adaptation still occurs in cells with a cAR1 receptor that cannot be phosphorylated. Apparently cells possess another mechanism of adaptation. The activation of adenylyl cyclase in *Dictyostelium* is complex. Our current model is that activation of cAR1 leads to the dissociation of the G-protein G2 into Gα2 and βγ (Kumagai *et al.*, 1991; Wu *et al.,* 1995). The βγ-complex stimulates adenylyl cyclase via a cytosolic protein CRAC that translocates to the membrane

(Insall *et al.*, 1994; Lilly & Devreotes, 1994; Lilly & Devreotes, 1995). In addition to the activation of the G-protein, a second pathway that is composed of the G-protein-independent activation of a MAP-kinase must be stimulated simultaneously (Segall *et al.,* 1995). Adaptation of receptor-stimulated adenylyl cyclase may be located at several places in this transduction pathway, including modification of cAR1 other than phosphorylation, or inactivation of any other component of the transduction pathway.

Dephosphorylation of the phospho-serines of cAR1 was thought to be related with the deadaptation of adenylyl cyclase, since this takes place with a half-life comparable to the half-life of deadaptation (Vaughan & Devreotes, 1988). We observed that CM1234 cells recover from this adaptation within 10 s after removal of the stimulus, which is much faster than deadaptation of control cells. This suggests that, although phosphorylation may not be required for adaptation, it could provide an additional mechanism to sustain the acquired state of desensitization of the adenylyl cyclase activating pathway. Thus a receptor must be dephosphorylated before it can become resensitized. These new observations lead us to propose an extension of the model for excitation and adaptation of adenylyl cyclase (Knox *et al.*, 1986). In this model, presented in figure 4, the receptor may adopt three states, each of which can exist in the free or ligand-bound state. Binding of ligand to the unoccupied receptor R leads to an active receptor conformation cAMP-R^* that causes excitation of adenylyl cyclase. This complex may follow two fates: either the ligand dissociates and the activated free receptor R^* rapidly falls back to the inactive form R, or the active receptor ligand complex converts to the adapted complex cAMP-R^A; this adapted form cannot elicit activation of adenylyl cyclase, and the presence of the ligand maintains the receptor in the adapted conformation. At this point the complex again may follow two routes: the ligand leaves the complex and the unoccupied adapted receptor R^A form reverts to its ground state R with a rate constant k_1, or the occupied adapted receptor becomes phosphorylated to the new form cAMP-R^A_P that is also adapted. When the ligand dissociates from the complex, the phosphorylated adapted receptor molecule R^A_P is slowly dephosphorylated with a rate constant k_2. In the absence of receptor phosphor-ylation, as in CM1234 cells, adaptation is still possible, but deadaptation is solely provided by the conversion of R^A to R which apparantly has a half-life of less then 10 s. When the receptor can be phosphorylated, deadaptation appears to be determined mainly by the rate of receptor dephosphorylation which shows a half-life of about 3 min. Although receptor phosphorylation is not required for adaptation of adenylyl cyclase, it still is expected to play a very important physiological role, because it

determines the kinetics with which cells become responsive after removal of the stimulus. Models on pulsatile cell aggregation predict that the kinetics of deadaptation has a major influence on the periodicity at which the pulsatile signals are relayed from cell to cell. The phenotype of the CM1234 mutant may well be explained by this phenomenon: Although excitation and adaptation of adenylyl cyclase are essentially unaltered, cell aggregation is chaotic with small aggregates being formed in an irregular way.

G-protein independent activation of the unusual adenylyl cyclase ACG by serpentine cAMP receptors in *Dictyostelium*

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Submitted

ABSTRACT

Recently the finding of two genes coding for adenylyl cyclase enzymes ACA and ACG in *Dictyostelium discoideum* was reported (Pitt *et al.*, 1992). ACA, the adenylyl cyclase that is present early in development, has been analyzed extensively in previous experiments. We report here the study of ACG, the adenylyl cyclase present in *Dictyostelium* spores. ACG activity can be stimulated 2,5- to ten-fold by the folic acid receptor and the cAMP surface receptor. The activation of the enzyme appears to be sensitive to temperature: at 0°C no activation was observed. The receptor mediated activation of ACG is inhibited by adenosine with a K_i=3.0 mM and caffeine (K_i=1 mM). Furthermore the cAMP analogue (Rp)-cAMPS has an antagonistic effect on the activation of ACG. The dcAMP mediated ACG response shows adaptation when the cells are exposed to prolonged stimuli. Experiments with cAMP receptor disruptants which overexpress ACG, showed that in absence of cAR1 and cAR3, dcAMP can not induce cAMP accumulation. However, in a cell line, overexpressing ACG in a G-protein gβ disruptant, a cAMP response can be induced by dcAMP and folic acid stimuli. The recently reported cytosolic factor CRAC, which is essential in the signaling response of ACA, is not involved in the activation of ACG.

INTRODUCTION

Cyclic AMP plays a central role in the developmental program of the slime mould *Dictyostelium*. In the unicellular stage, the individual cells are attracted towards their bacterial food source by means of chemotaxis to folic acid that is secreted by bacteria. Upon food depletion the role of chemoattractant is taken over by cAMP, which triggers a developmental program. Pulses of cAMP secreted by the starving cells induce chemotaxis in the surrounding cells, which also secrete cAMP to relay the signal. In this way cells aggregate and form a multicellular slug, that finally develops into a fruiting body, composed of dead stalk cells and viable spores. Detection of cAMP occurs by four different cell surface cAMP receptors (cAR1-4), all having their own typical time period of expression. These cAMP receptors have a structure that consists of seven putative membrane spanning domains, which is characteristic for G-protein coupled receptors. In the early stage of development, cAMP stimulation of cAR1 provides the activation of the heterotrimeric G-protein Gα2βγ (Klein *et al.*, 1988; Saxe *et al.,* 1991a; Pupillo *et al.*, 1992; Insall *et al.,* 1994a), which results in activation of adenylyl cyclase, guanylyl cyclase and phospholipase C (Kumagai *et al.*, 1989; Okaichi *et al.*, 1992). Processes like the activation of G-box-binding factor GBF, receptor phosphorylation and Ca^{2+} uptake, are mediated also mainly by cAR1, but these events occur independently from the G-protein (Milne *et al.*, 1995; Schnitzler *et al.*, 1995). Receptor stimulation does not lead to an endless activation of adenylyl cyclase. Instead, the cAMP levels are regulated by an adaptation mechanism, that makes the cAMP receptor temporarily insensitive to cAMP stimuli, which results in well-tuned pulses of cAMP production (Devreotes and Steck, 1979).

In *Dictyostelium* two genes have been cloned that encode for adenylyl cyclase, designated ACA, the cyclase expressed mainly during cell aggregation, and ACG that is present during spore germination (Pitt *et al*., 1992). ACA with a molecular

size of 160 kD, resembles the mammalian adenylyl cyclases, having two domains each spanning the membrane six times, and two 40 kD cytosolic regions. The cytosolic protein CRAC serves as connection for the signal transfer from G-protein to ACA. Mutant strains *Synag7* and *dagA*$^-$, which lack CRAC, are unable to activate ACA and can not aggregate nor develop (Theibert and Devreotes, 1986). These defects are all rescued when the mutant cells are transformed with cDNA encoding for CRAC.

The proposed structure of ACG predicts a membrane-bound protein of 98 kD, with a large extracellular domain, one transmembrane segment and a single intracellular catalytic domain, similar to the topology of membrane-bound guanylyl cyclases. Expression of ACG in cells lacking ACA proves that ACG is indeed an adenylyl cyclase.

Cells with a disrupted ACA gene (*aca*$^-$) do not form aggregates, but persist in the monolayer stage; developmental gene expression is blocked, which demonstrates that ACA is essential for development. Application of pulses of extracellular cAMP to these cells rescues the mutant phenotype, indicating that intracellular cAMP is not essential for development (Pitt *et al.*, 1993). Previous experiments showed that expression of ACG in *aca*$^-$ cells partly restores cell aggregation and fruiting body formation, implicating that ACG incorporates the ability of providing extracellular cAMP in a manner that allows the stimulation of aggregative gene expression as well as chemotaxis (Pitt *et al.*, 1992). Aggregate formation in *Dictyostelium discoideum* requires pulsatile production and secretion of cAMP, which enables the amoeba to move towards the origin of the cAMP wave by determining the highest concentration in the cAMP gradient (Newell, 1982). Also several forms of aggregative gene expression require the pulsatile production of cAMP (Schaap *et al.*, 1986). Modulation of adenylyl cyclase activity via activation and adaptation of surface cAMP receptors is probably essential for pulsatile cAMP production.

Pitt *et al.* (1992) suggest that ACG is a constitutively active adenylyl cyclase, which is not activated by a G-protein or the cAMP receptor at reduced temperatures, in contrast with ACA. The question then arises: how are *aca*$^-$ cells rescued by ACG if cell aggregation and development requires pulsatile production and secretion of cAMP.

In this paper we describe the activation of ACG by extracellular cAMP, studied in the mutant strain, *aca*$^-$*/ACG*$_{act\text{-}15}$. In this cell line ACG remains under transcriptional control of the *Dictyostelium* actin 15 promoter, and it is expressed during both the vegetative and the developmental stage (Pitt *et al.*, 1992).

We observed that ACG is activated about ten-fold by extracellular cAMP and almost three-fold by folic acid. In this signal transduction from receptor to ACG, G-proteins and CRAC are not involved. We conclude that the G-protein coupled receptor cAR1 activates ACG via a G-protein independent mechanism.

MATERIALS & METHODS

Materials

[2,8-^{3}H]cAMP (50 Ci/mmol) was obtained from Amersham, 2 -deoxy-cAMP was from Sigma. DTT, adenosine, cAMP, ATP and GTPγS were from Boehringer Mannheim, (Rp)-cAMPS was a kind gift of Dr. B. Jastorff (University of Bremen, Germany). Caffeine was obtained from Aldrich-Europe, EDTA was from Merck. Nuclepore polycarbonate filters were from Costar.

Vector construct

Plasmid pMB15, an extrachromosomal cAR1 expression vector, was obtained by insertion of the 1.3 kb BamH1/BglII fragment from plasmid p6B containing the cAMP receptor gene cAR1 (Klein *et al.*,

1988) in the BglII site of vector pMB3. This is the pBlueScript S/K vector with a 1.3 kb XbaI/HindIII insert of the blasticidin resistance gene bsrΔBamHI/BglII from plasmid pBSR2 (Sutoh, 1993), the partial digested 2.9 kb HindII/ClaI fragment containing the origin of replication for *Dictyostelium discoideum* from plasmid p155d1(Hughes *et al.*, 1994) and the 1.2 kb BamHI/BclI fragment from plasmid BS18.2H3 containing the actin15 promoter and 2H3 terminator (Kumagai *et al.*, 1989).

Cell transformations and cell culture The *g*β⁻*/ACG* and *car1⁻/car3⁻/ACG* cells were made by transformation of *g*β⁻ cells (strain LW6, Lilly *et al.*, 1993) and *car1⁻/car3⁻* cells (strain RI4, Insall *et al.*, 1994a) with plasmid pGSP1, which contains the ACG coding region between the actin 15 promoter and the 2H3 terminator and a neomycin resistance gene (Pitt *et al.*, 1992) The *g*β⁻*/cAR1/ACG* cells were made by transformation of *g*β⁻ cells (strain LW6) with plasmid pMB15 which contains the cAR1 coding region between the actin 15 promoter and the 2H3 terminator and a blasticidin resistance gene. Subsequently the resulting cell line *g*β⁻*/cAR1* was transformed with plasmid pGSP1. Briefly, the cells were harvested in the early logarithmic phase, washed and resuspended at 0°C in electroporation buffer (10 mM NaH_2PO_4/Na_2HPO_4, pH 6.1, 50 mM sucrose) at a cell density of 2.10^7 per ml. In an electroporation cuvette 400 µl cells were mixed with 5 µg plasmid, cells were pulsed (50 µF, 500 V, 13 ohms, estimated field strength 2.5 kV cm^{-1}) and left to recover for 10 minutes. Cells were transferred to petri dishes, 4 µl 0.1 M $CaCl_2$ and 4 µl 0.1 M $MgCl_2$ was added. After 15 minutes 6 ml HG5 was added, 6 hours later this medium was renewed by HG5 medium containing the appropriate antibiotic (10 µg/ml G418 for *car1⁻/car3⁻/ACG* and *g*β⁻*/ACG* cells, 10 µg/ml G418 and 10 µg/ml blasticidin for *g*β⁻*/cAR1/ACG* cells). HG5 medium is made according to HL5 medium (Ashworth and Watts, 1970), with addition of 10 g glucose per liter instead of maltose.

Wild-type AX3, LW6 and RI4 cells were grown at 22°C in HG5 medium; *aca⁻/ACG, crac⁻/ACG* cells (Pitt et al., 1992) *g*β⁻*/ACG* and *car1⁻/car3⁻/ACG* cells were grown in HG5 medium containing 10 µg/ml G418, *g*β⁻*/cAR1/ACG* cells were grown in HG5 medium containing 10 µg/ml G418 and 10 µg/ml blasticidin. Cells were harvested in the late logarithmic phase and starved for 4 hours on a rotary shaker in 10 mM KH_2PO_4/Na_2HPO_4 pH 6.5 (PB) at a cell density of 10^7 per ml. Cells were washed twice, resuspended in PB and aerated for at least 15 minutes at 22°C.

cAMP accumulation in vivo
Washed cells were resuspended in PB at a density of $5x10^7$ per ml. Forty five µl cell suspension were mixed with 5 µl stimulus (5 µM dcAMP and 10 mM DTT final concentration, or 10 µM folic acid final concentration). For the measurement of total cAMP, the reaction was stopped at indicated time points by adding 50 µl 3.5% (v/v) $HClO_3$. For the measurement of extracellular and intracellular cAMP cells were spun down for 5 s at 16000 g. Extracellular and intracellular cAMP were separated by transferring the supernatant to 50 µl 3.5% (v/v) $HClO_3$, and resuspending the pellet in 50 µl PB and an equal volume of 3.5% (v/v) $HClO_3$. Solutions were neutralized by adding 25 µl $KHCO_3$ (50% saturated at 20°C), and the cAMP content was measured by isotope dilution, using the regulatory subunit of cAMP-dependent protein kinase type I (Van Haastert, 1984). Because cAMP as a stimulus would interfere with the assay of cAMP as the product of adenylyl cyclase, an analogue dcAMP is used as a stimulus, which binds effectively to surface receptor, but poorly to the binding protein used in the assay.

Adenylyl cyclase activity in vitro

Cells were starved for 5 hours, washed and resuspended in PB at a density of $1.5.10^8$ per ml, mixed 1:1 with ice-cold lysis buffer (20 mM Tris.Cl pH 8.0, 6mM $MgCl_2$), and lysed at 0°C by rapid elution through a double Nuclepore filter (pore size 3.0 µm). To determine adenylyl cyclase activity *in vitro* after receptor stimulation *in vivo*, cells were stimulated with 5µM dcAMP and 10 mM DTT (final concentration) stored on melting ice for 1 minute and mixed with an equal volume of lysis buffer and lysed. Lysates were assayed immediately, the adenylyl cyclase activity assay contained 20 µl lysate, mixed with 20 µl assay buffer (10 mM Tris.Cl pH 8.0, 3mM $MgCl_2$, 20 mM DTT, 1 mM ATP and optionally 10 mM $MnSO_4$). The assay was performed at 22°C or 0°C and terminated at 0 min, 5 min and 15 min by adding 10 µl 0.5M EDTA followed by boiling for 2 min. Cyclic AMP levels were determined by isotope dilution as described above.

RESULTS

Activation of ACG by surface cAMP receptors

To determine how ACG rescues *aca⁻* cells, we examined stimulation of ACG activity by the receptor agonist dcAMP. Addition of dcAMP to *aca⁻/ACG* cells resulted in a ten-fold increase of intracellular cAMP levels (fig. 1A). The kinetics of the response resembles the kinetics of dcAMP mediated ACA activation in wild-type AX3 cells (fig. 1B): intracellular cAMP levels start to increase at 15 seconds after stimulation, peaks after 1-2 min and basal levels are

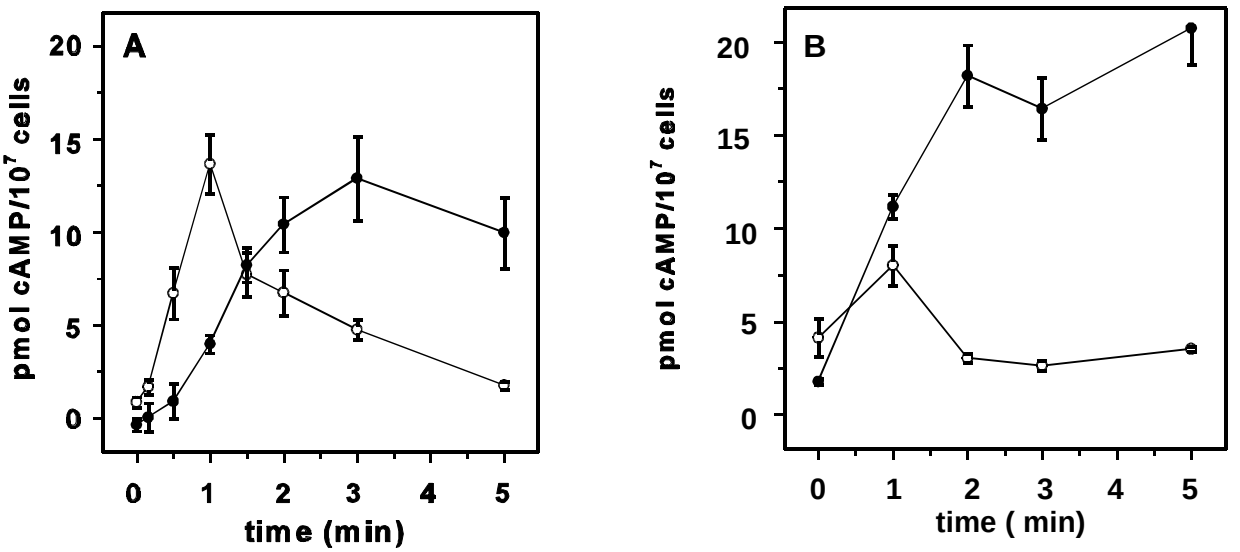


Figure 1. *Response of aca⁻/ACG cells to dcAMP stimulation.* Cells, starved for 5 h, were washed and stimulated with 5 µM dcAMP, 5 mM DTT at t=0 s. At indicated time points samples were taken, separated into intracellular and extracellular compartments, and reactions were stopped by addition of one volume 3.5% perchloric acid. After neutralization with $KHCO_3$, cAMP levels were measured. Data represent means of two experiments in triplicate. ○: intracellular cAMP,●: extracellular cAMP. Panel A: *aca⁻/ACG* cells, panel B: control cells AX3.

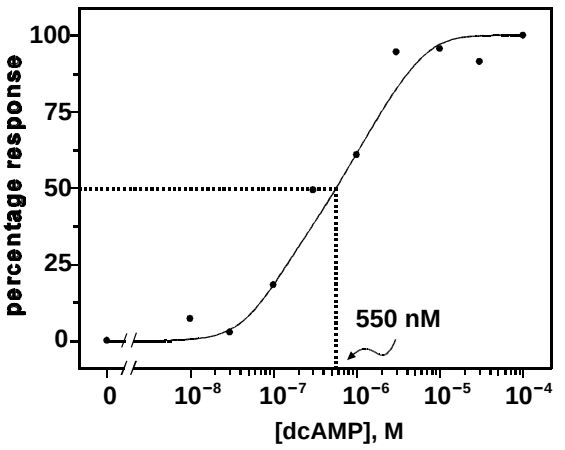


Figure 2. *Dose response curve of ACG to stimulation with dcAMP.*
Cells (*aca⁻/ACG*) were starved for 5 h, washed and stimulated with different concentrations of dcAMP at t=0 s. Intracellular cAMP levels were measured by separating cellls from supernatant at t=60 s, and stopping the reaction by addition of one volume 3.5% perchloric acid to the cell pellet. Data represent two experiment in triplicate

recovered after 5-10 min (Devreotes & Steck, 1979). Secretion of cAMP was observed for both *aca⁻/ACG* cells and wild-type AX3 (fig. 1).

A dose response of dcAMP mediated stimulation of intracellular cAMP levels in *aca⁻/ACG* cells reveals a significant response at 10^{-7} M dcAMP. A half maximal response is induced at 550 nM dcAMP, whereas saturation occurs at about 10^{-5} M dcAMP (fig. 2); these concentrations are approximately 6.5 times higher than in wild-type cells (Theibert *et al.*, 1986).

Inhibition by adenosine and caffeine

The cAMP signaling response via ACA in wild-type cells is inhibited by adenosine. To determine the influence of adenosine on the activation of ACG, we measured in the presence of different adenosine concentrations the cAMP response of *aca⁻/ACG* cells to a dcAMP stimulus of 200 nM or 2 µM and 10 mM DTT. Half-maximal inhibition occurred at 3.0 mM adenosine (fig. 3) at both stimulus concentrations (200 nM dcAMP not shown). The *aca⁻/ACG* cells are approximately 10 times less sensitive to the inhibitory action of adenosine than wild-type cells (Theibert & Devreotes, 1984).

In wild-type cells, caffeine is another inhibitor of ACA stimulation. We observed that also caffeine inhibited the cAMP response in *aca⁻/ACG* cells with half-maximal inhibition at 1 mM caffeine (fig. 3) and a 5 µM dcAMP stimulus. Analogous to adenosine inhibition, we observed for caffeine a sensitivity in *aca⁻/ACG* cells 1.7 times lower than in wild-type cells (Theibert & Devreotes, 1983).

Activation of ACG is temperature dependent

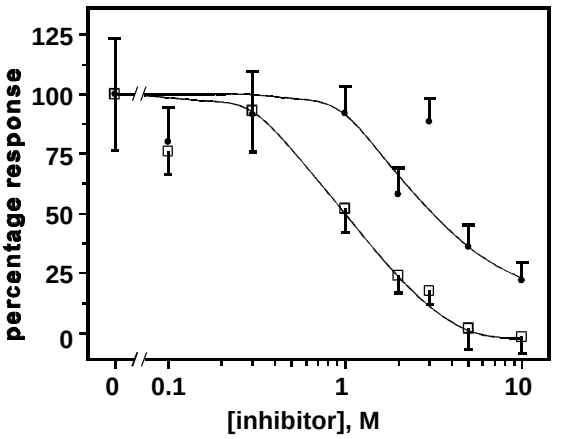


Figure 3. *Adenosine and caffeine inhibition of dcAMP mediated cAMP response.*
Cells (*aca⁻/ACG*) were starved for 5 h and washed with PB; 30 µl of the cell suspension was mixed with a 20 µl stimulus. Adenosine inhibition was measured at a stimulus of 2 µM or 0.2 µM dcAMP, 10 mM DTT (final concentration) and different concentrations of adenosine. Caffeine inhibition was measured at a stimulus of 5 µM dcAMP, 10 mM DTT (final concentration) with addition of caffeine. Intracellular cAMP was measured after 1 min of incubation. ●: adenosine inhibition of cells stimulated with 2 µM dcAMP, □:caffeine inhibition at a 5 µM dcAMP stimulus.

Shortly after the intracellular increase of cAMP upon dcAMP stimulation, wild-type AX3 cells secrete cAMP to the extracellular medium, a process called cAMP-relay, which is temperature dependent (Van Haastert, 1984, 1987a). We investigated the effect of temperature in *aca⁻/ACG* cells on the accumulation of intra- and extracellular cAMP levels. Surprisingly, the *aca⁻/ACG* cells stimulated with dcAMP at 0°C cAMP show no detectable cAMP synthesis (fig. 4A). In a time course in which the cells were chilled to 0°C at different time points after stimulation at 22°C, we observed immediate termination of the intracellular cAMP production (fig. 4A) and cAMP secretion (fig. 4B). Thus the effect of temperature on ACG activation is more dramatic than on ACA activation: in *aca⁻/ACG* cells both the activation of ACG and the secretion of cAMP are temperature dependent.

The observed temperature sensitivity of the dcAMP mediated cAMP response in *aca⁻/ACG* cells can be located in the adenylyl cyclase itself or more upstream, between receptor and ACG. In order to investigate the temperature sensitivity of ACG itself, we measured the activity *in vitro*. In *aca⁻/ACG* cells, ACG shows maximal *in vitro* activity in the presence of Mn^{2+} ions at 22°C (table 1). This activity is sensitive to temperature; at 0°C, the activity is reduced eight-fold. The ACG activity in the presence of Mg^{2+} ions at 22 °C is 72% compared to the activity in presence of Mn^{2+}. A four-fold reduction of this activity is observed when measurements are performed at 0°C. Previously, similar experiments have been performed with lysates of wild-type NC4 cells, showing that the ion- and temperature sensitivity of ACA is not very different from that of ACG (table 1) (Khachatrian *et al.*, 1987; Van Haastert *et*

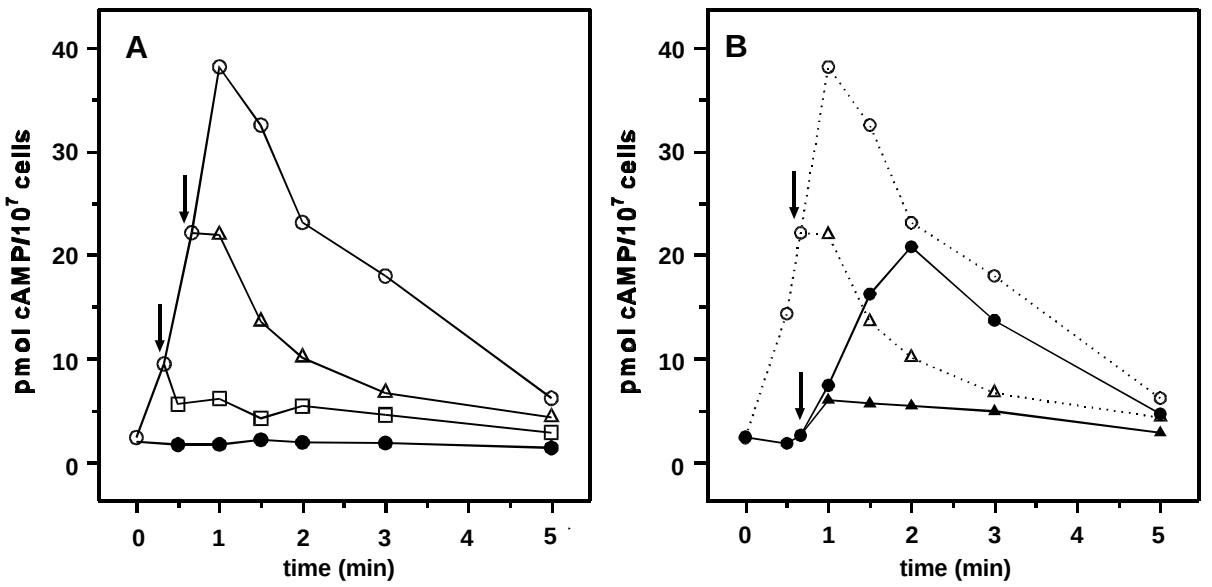


Figure 4. *Effect of temperature on ACG activity.*
Cells (*aca⁻/ACG*) were starved for 5 h and washed. Panel A. Intracellular cAMP concentration of cells stimulated with 5 µM dcAMP and placed on ice at different time points. Control series were measured at a fixed temperature (○ : 22°C, ●: 0°C); time courses were measured with a change in temperature (□ and). Arrow: time point at which a sample is switched from 22°C to 0°C. Panel B. Intracellular (○, , dotted lines) and extracellular (●,▲, solid lines) cAMP concentrations of dcAMP stimulated samples placed on ice at indicated time points (arrow).

al., 1987a). Thus, the *in vitro* activity of ACG is comparable to that of ACA at 22°C as well as at 0°C, whereas the absence in ACG activation after receptor stimulation at 0°C differs strongly from the observations on ACA activation. This suggests that the observed absence of receptor-mediated activation of ACG at 0°C cannot only be explained by the reduced *in vitro* activity of ACG at 0°C. We conclude that the transduction pathway between receptor and adenylyl cyclase is very temperature sensitive for ACG and relatively insensitive for ACA.

Adaptation of ACG stimulation

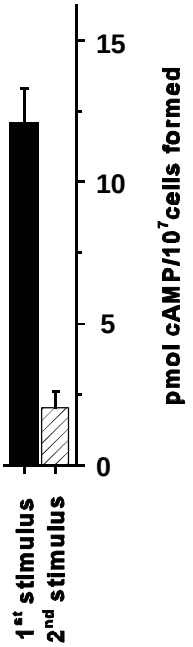


Figure 5. *Adaptation of dcAMP mediated ACG response.*
Cells (*aca⁻/ACG*), starved for 5 h, were washed and stimulated with 5 µM dcAMP, 10 mM DTT at t=0 s, and samples were taken at different time points. At t=15 min the cells were chilled on ice for 1 min, spun down at 300 g and washed with 1 ml ice-cold PB. The cells were resuspended in the original volume PB (22°C) and a second stimulus was applied. Intracellular cAMP was measured. Data represent the increase of cAMP production after the first (solid bar) and second stimulus (open bar) as measured in two experiments in triplicate.

The transient character of the cAMP accumulation after receptor stimulation in *aca⁻/ACG* cells could be the consequence of adaptation. In wild-type cells, prolonged exposure to a dcAMP stimulus makes the cell incapable to respond during several minutes to a second stimulus of the same concentration (Dinauer *et al.,* 1980). We tested whether the ACG response is mediated by adaptation. Therefore a dcAMP prestimulus was given to *aca⁻/ACG* cells during 15 minutes, the cells were washed and the response to a second dose of dcAMP was measured. The first stimulus induced a transient cAMP accumulation, however, the cells did not react to the second stimulus (fig. 5). A shorter incubation period with the first stimulus (7 min) leads to a partial

Table 1. *Adenylyl cyclase activity in cell lysates*
Cells were starved for 5 h, lysed, and assayed for adenylyl cyclase activity at 0°C or 22°C with Mn^{2+} or Mg^{2+}. Experiments were performed three times in triplicate, and compared with adenylyl cyclase activity *in vitro*, measured in *Dictyostelium* strain NC4 (Van Haastert *et al.*, 1987a). The activity of ACG in the presence of Mn^{2+} at 22°C (100%) corresponds with 389 pmol cAMP (mg protein)$^{-1}$.min^{-1}, which is a 10 times higher activity than ACA under the same conditions.

		aca⁻/ACG	NC4
22°C	Mn^{2+}	100 ± 5	100 ± 4
	Mg^{2+}	72 ± 19	80 ± 5
0°C	Mn^{2+}	26 ± 6	14 ± 1
	Mg^{2+}	12 ± 2	1.2 ± 0.1

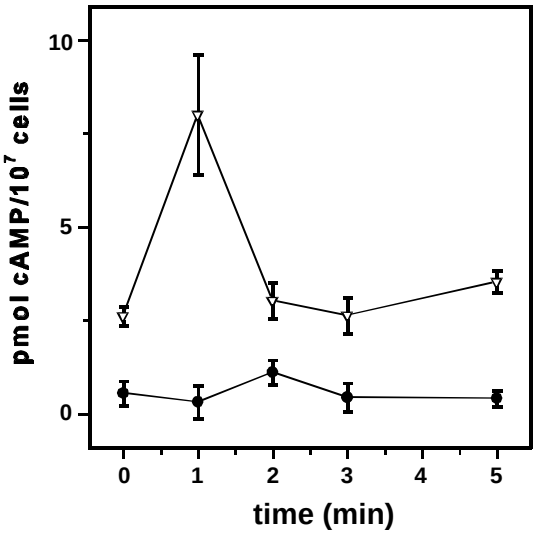


Figure 6. *cAMP response in car1⁻/car3⁻/ACG cells*.
Starved *car1⁻/car3⁻/ACG* cells and control cells (AX3) were washed and stimulated with 5 µM dcAMP, 10 mM DTT and monitored for total cAMP for the next 5min. ●: *car1⁻/car3⁻/ACG* (total cAMP), : AX3 (intracellular levels).

adaptation of the dcAMP mediated cAMP response (data not shown). These results demonstrate that dcAMP-mediated activation of ACG is subject to adaptation, which is similar to receptor mediated activation of adaptation of ACA.

ACG is coupled to the cAMP receptor cAR1 and/or cAR3

The response to a dcAMP stimulus suggests that the cAMP receptor is involved in the activation of ACG. *D. discoideum* has four different cAMP receptor genes, designated cAR1-cAR4, of which the receptor isoforms cAR1 and cAR3 are both involved in the activation of ACA (Pupillo *et al.*, 1992). To obtain information about the identity of the cAMP receptor involved, we transformed a *car1⁻/car3⁻* cell line with the ACG expression vector plasmid pGSP1 (Pitt *et al.*, 1992). The mutant cell line was tested for cAMP response. Figure 6 shows that these ACG-overexpressing cells, lacking the cAR1 and cAR3 receptors, do not show an increase in cAMP synthesis upon stimulation with dcAMP/DTT. This suggests that ACG is stimulated by cAMP via cAR1 and/or cAR3.

(Rp)-cAMPS antagonism of dcAMP induced ACG response.

We investigated the action of the cAMP analogue (Rp)-cAMPS in *aca⁻/ACG* cells. This cAMP antagonist competes with the binding of cAMP with receptor isoforms cAR1-3; the dcAMP stimulation of ACA is inhibited by (Rp)-cAMPS. The cAMP-analogue itself, however, does not activate ACA (Van Haastert, 1987b). Incubation of *aca⁻/ACG* cells with

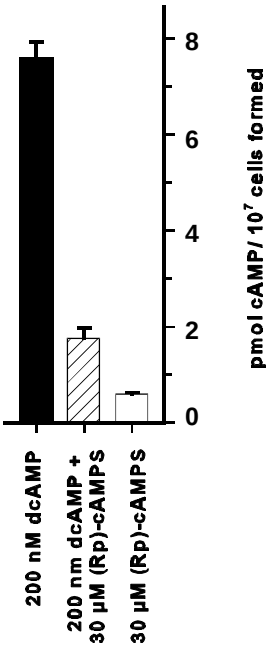


Figure 7. *(Rp)-cAMPS antagonism of dcAMP mediated response*.
Cells (*aca⁻/ACG*) were starved 5 h and washed. The increase of intracellular cAMP concentration was measured 60 s after stimulation with 200 nM dcAMP (closed bar) or 200 nM dcAMP + 30 µM (Rp)-cAMPS (hatched bar), compared with cells stimulated with 30 µM (Rp)-cAMPS (open bar).

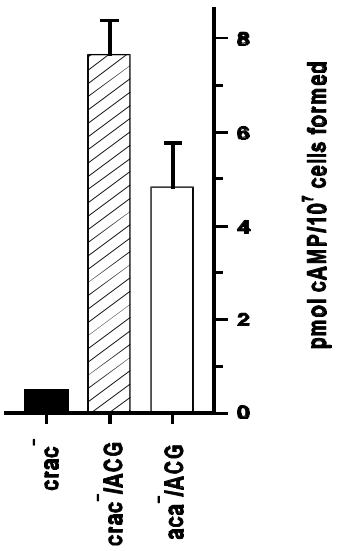


Figure 8. *dcAMP mediated cAMP response in crac⁻/ACG cells.*
Cells were starved, washed, and stimulated at t=0 s with 5 µM dcAMP and 10 mM DTT. Increase in cAMP levels was measured in *crac⁻/ACG* cells (hatched bar) and in control cells *aca⁻/ACG* (open bar) 60 s after stimulation. The levels are compared with the cAMP levels measured in crac⁻ cells (solid bar) as described by Insall *et al.* (1994b).

dcAMP/(Rp)-cAMPS leads to a reduction of 80% of the adenylyl cyclase response, compared to the normal dcAMP mediated response (fig. 7). As a control, cells were incubated with (Rp)-cAMPS without dcAMP, which does not show a detectable increase of cAMP synthesis. We conclude that, like in wild-type cells, (Rp)-cAMPS is an antagonist of the dcAMP stimulated activation of ACG in *aca⁻/ACG* cells.

Involvement of CRAC with cAMP mediated ACG response

In wild-type cells the cytosolic protein CRAC is involved in the transfer of the signal from the stimulated receptor-G-protein complex to ACA (Lilly & Devreotes, 1994; Insall *et al.*, 1994b). We investigated whether receptor activation of ACG is also mediated by CRAC. For this purpose a mutant cell line BW3 was constructed, which overexpresses ACG in a *crac⁻* background. Stimulation of these *crac⁻/ACG* cells with dcAMP induced an increase of cAMP levels that is similar to the cAMP response induced by dcAMP in *aca⁻/ACG* cells (fig. 8). This observation indicates that CRAC does not participate in the signal transduction from cAMP receptor to ACG.

ACG is activated by folic acid receptors

The resemblance of ACG with membrane-bound guanylyl cyclase made ACG a candidate to be a guanylyl cyclase itself. One of the differences between *Dictyostelium* guanylyl cyclase and adenylyl cyclase ACA activity, is that guanylyl cyclase can be stimulated by folic acid in vegetative cells, whereas ACA not (Devreotes, 1983). We examined a possible activation of ACG by folic acid. Stimulation of vegetative *aca⁻/ACG* cells with folic acid indeed leads to a 2-3 fold increase of cAMP (fig. 9A). The observed folic acid mediated response is temperature dependent: at 0°C no cAMP response was observed (data not shown). To rule out the possibility of folic acid acting indirectly via the cAMP receptor, the experiment was repeated with *car1⁻/car3⁻/ACG* cells. In these cells we also observed a 2-3 fold increase of cAMP production upon addition of folic acid (fig. 9B). The same cells did not show cAMP accumulation after folic acid stimulation when the experiment was performed at 0°C instead of 22°C (fig. 9B). Control experiments with the *car1⁻/car3⁻* parental strain revealed no cAMP response to folic acid stimulation (fig. 9B).

Involvement of G-protein with ACG response in Dictyostelium

ACA *in vitro* is stimulated by GTPγS; addition of GTPγS to cell lysates within a few seconds after cell lysis gives an up to 17-fold increase of ACA activity. Preincubation with cAMP prior to lysis in

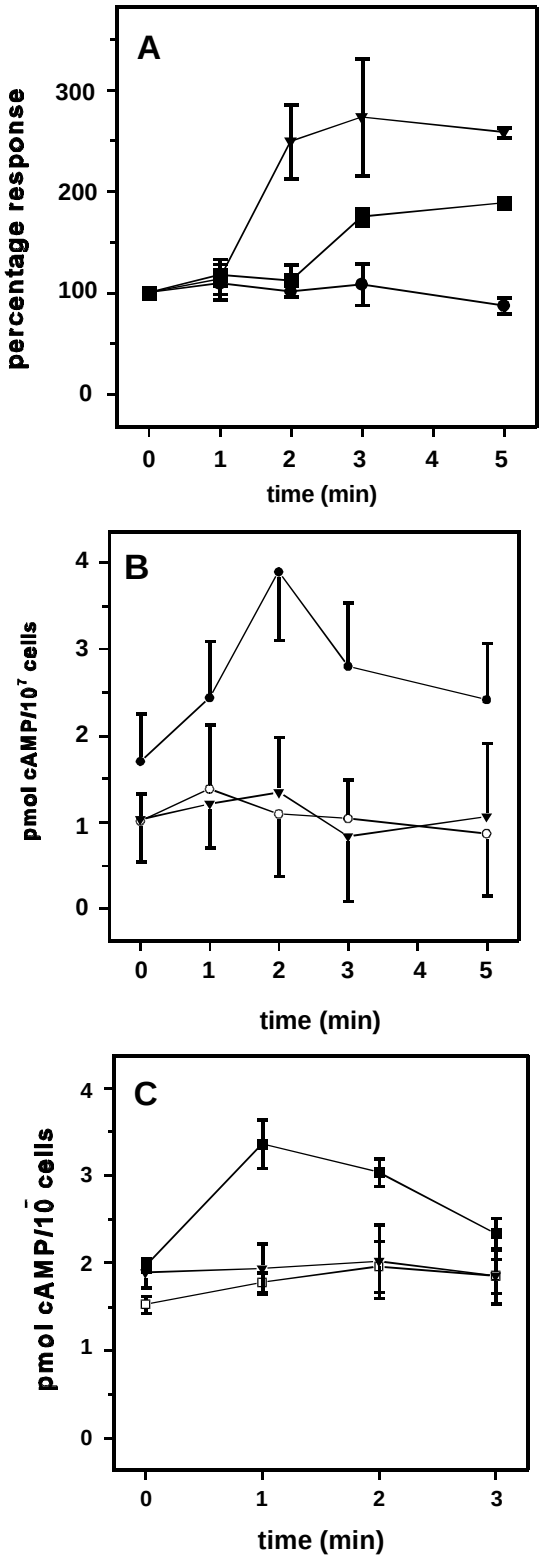


Figure 9. *Folic acid and dcAMP mediated cAMP response in car1⁻/car3⁻/ACG and gβ⁻/ACG cells*.
Folic acid stimulations were performed with vegetative cells, obtained from exponentially growing cells, which were starved for 30 min and washed. At t=0 s cells were stimulated with 10 µM folic acid and the total cAMP was measured during several minutes. Stimulations with dcAMP were performed with aggregation competent cells (*gβ⁻/cAR1/ACG),* obtained as follows: cells were starved for 5 h, with 100 nM cAMP pulses from t=2h till t=5h. Cells were washed and stimulated at t=0 s with 10 µM dcAMP (final concentration). Panel A. cAMP levels in folic acid stimulated *aca⁻/ACG* cells (▼); folic acid stimulated *gβ⁻/ACG* cells (■); and folic acid stimulated *gβ⁻* cells (●). Panel B: cAMP levels after folic acid stimulation in *car1⁻/car3⁻/ACG* cells at 22°C (●) and 0°C (○) and *car1⁻/car3⁻* cells at 22°C (▼). Panel C: cAMP levels in dcAMP stimulated *gβ⁻/cAR1/ACG* (■), *gβ⁻/cAR1* (□) and *gβ⁻/ACG* ( ) cells.

the presence of GTPγS increases the activation up to 32-fold (Theibert & Devreotes, 1986; Van Haastert *et al.*, 1987a). In *aca⁻/ACG* cell lysates, addition of GTPγS directly after cell lysis showed no significant increase in ACG activity (106±11.9%, n=3, 0.5<p<0.1), suggesting that stimulation of ACG may not be mediated by a G-protein.

To obtain direct evidence for the contribution of G-proteins to the receptor activation of ACG, we tested ACG activity in the absence of G-protein. Therefore we transformed a *Dictyostelium gβ⁻* cell line with the plasmid pGSP1, and because *gβ⁻* cells have very low levels of cAMP receptors (Wu *et al.*, 1995), also with plasmid pMB15 for cAR1 overexpression, and analyzed the mutant cell line (*gβ⁻/cAR1/ACG*) for cAMP response. Upon stimulation with dcAMP, these cells show a significant increase of cAMP production (fig. 9C). One minute after dcAMP stimulation the basal cAMP level

has increased almost 2-fold, and slowly declines. The parental line, which is *g*β⁻*/cAR,* initially shows no significant rise of the cAMP concentration upon receptor stimulation. Stimulation of vegetative *g*β⁻*/ACG* cells with folic acid also induces a significant increase in cAMP production (fig. 9A). Five minutes after stimulation a two-fold increase of basal cAMP levels is achieved. Folic acid induces no cAMP response in the parental strain *g*β⁻ (fig. 9A).

DISCUSSION

Signal transduction during cell aggregation and development of the slime mould *Dictyostelium discoideum* is initiated by the production and secretion of cAMP. In order to sense this first messenger, *Dictyostelium* has a set of four cAMP receptors available at different overlapping time periods in development (Saxe *et al.*, 1991a). For the potential production of cAMP, the *Dictyostelium* genome reveals two genes that code for adenylyl cyclase enzymes. The gene expressed early in the developmental program, codes for ACA, the cAMP synthesizing protein that is responsible for the majority of cAMP-mediated events reported thus far (Pitt *et al.*, 1992). The messenger RNA for ACG, the second adenylyl cyclase, is detectable at the final stage of the developmental program in the fruiting bodies and peaks during the first 1.5 hours of spore germination.

Mutants lacking ACA activity do not enter the developmental program; however, pulses of extracellular cAMP given to *aca*⁻ cells have proven to be sufficient to guide the mutant cells into development (Pitt *et al.*, 1993). Expression of ACG in these mutants as well leads to a rescue of the aberrant phenotype (Pitt *et al.*, 1992). ACG does not have the tertiary structure of the known adenylyl cyclases with two domains of six transmembrane segments and two catalytic domains; instead, it resembles more the membrane bound guanylyl cyclase with a large N-terminal extracellular domain, one trans-membrane segment and one intra-cellular catalytic domain. ACG is a genuine adenylyl cyclase, because overexpression of ACG in cells leads to an increase of the *in vitro* cAMP synthesis, while the cGMP production remains unchanged (Pitt *et al.*, 1992). Our experiments show that ACG can be stimulated by surface receptors. Although some aspects of the receptor-mediated activation of ACA and ACG are similar, the transduction pathway from receptor to ACG appears to be very different from that of receptor to ACA, and also different from that of receptor to guanylyl cyclase (GC). The results of the experiments are summarized in table 2.

Stimulation of *aca*⁻*/ACG* cells with dcAMP leads to an increase of intracellular and extracellular cAMP, in analogy with activation of cells expressing ACA. One minute after administration of the stimulus, intracellular cAMP levels have increased ten-fold and subsequently decrease to basal values. Activation of GC is much faster, with maximal cGMP levels at 10 s after stimulation and a return to basal levels within 30 s.

The receptor that mediates the activation of ACG shows characteristics of a classical cAMP receptor. Incubation of *aca*⁻*/ACG* cells with a mixture of dcAMP and the cAMP analogue (Rp)-cAMPS leads to a reduced cAMP response. In wild-type cells, (Rp)-cAMPS was found to bind to all forms of the cAMP receptor, which did not result in the activation of ACA or GC, probably because it lacks the specific three-dimensional structure, that is necessary to activate a cAMP receptor (Van Haastert

Table 2. *Comparison of regulation of ACA, ACG and GC.*

	ACA	ACG	GC
mol wght	160 kD	98 kD	unknown
cAR1	+	+	+
cAR3	+	+	+
folic acid receptor	-	+	+
CRAC	+	-	-
G-protein	+	-	+
activation at 22°C	+	+	+
activation at 0°C	+	-	±
caffeine K_i (mM)	0.6	1	0
adenosine K_i (mM)	0.3	3.0	~1*
Rp-cAMPS	antagonist	antagonist	antagonist

*) The cGMP response is only partly inhibited by adenosine: maximally 45% inhibition at 5 mM, halfmaximal inhibition at 1 mM adenosine (Van Haastert, 1983b)

& Kien, 1983; Van Haastert, 1987b). Cyclic AMP-induced activation of ACA and GC is mediated predominantly by the surface receptor cAR1, while cAR3 can take over this function when the expression of cAR1 is inhibited (Pupillo *et al.*, 1992, Insall *et al.,* 1994a). Stimulation of ACG by dcAMP appears to be mediated by the same surface receptors cAR1/cAR3. Overexpression of ACG in cells with a disruption of both cAR1 and cAR3 genes leads to cells with enhanced basal adenylyl cyclase activity, which however cannot be stimulated by cAMP *in vivo.*

Prolonged exposure to a dcAMP stimulus results in adaptation of the ACG response, a reaction that resembles the adaptation observed for ACA and guanylyl cyclase in wild-type cells (Devreotes & Steck 1979, Van Haastert & Van der Heijden, 1983). The mechanism of adaptation enables the cell to recapitulate and to determine the origin of the wave of cAMP in order to move into the direction of the increasing gradient. Adaptation of ACA is independent of the stimulation of its adenylyl cyclase activity (Theibert & Devreotes, 1983) and depends on the expression and activation of cAR1 (Pupillo *et al.*, 1992).

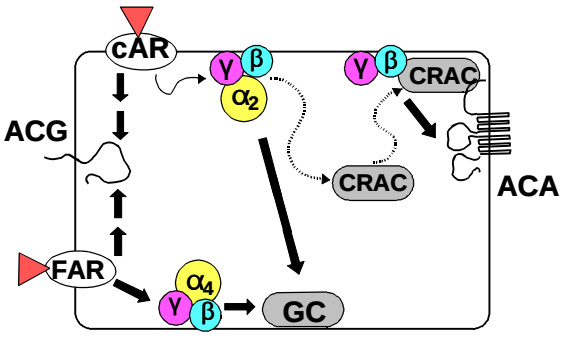
Receptor-mediated activation of ACA is rather specifically inhibited by caffeine. This drug does not inhibit the activation of GC or the receptor-mediated uptake of Ca^{2+} ions (Brenner & Thoms, 1984; Milne & Coukell 1991). Stimulation of *aca⁻/ACG* cells with dcAMP and increasing concentrations of caffeine leads to a decrease in the ACG response, demonstrating that ACG activation is sensitive to the inhibitory effect of caffeine. The sensitivity of ACG to caffeine is not very much different compared to ACA in wild-type cells; ACG needs a 1.7-fold higher caffeine concentration to induce a 50% inhibition.

The data discussed thus far show that detection of the dcAMP signal and adaptation to this signal is essentially

identical for stimulation of ACA and ACG; all these effects are mediated predominantly by cAR1. However, significant differences are noticed when the transduction of these signals is investigated. Stimulation of ACG by dcAMP is half-maximal (K_A) at about 50 nM; stimulation of ACA requires about ten-fold lower concentrations and occurs with a K_A of 5 nM. Since the K_D of cAR1 is about 50-100 nM, this suggests that spare receptors exist for the activation of ACA, but not for the stimulation of ACG.

Adenosine is a drug that strongly inhibits the receptor-mediated activation of ACA in wild-type cells (Theibert & Devreotes, 1984). Adenosine inhibits the binding of cAMP to intact cells, hereby blocking the activation of adenylyl cyclase; the kind of inhibition (competitive versus non-competitive or mixed competitive) depends on the developmental stage (Van Haastert, 1983b; Theibert & Devreotes, 1984). Adenosine also has a significant inhibitory effect on the activation of ACG, with a lower sensitivity compared to ACA in wild-type cells. The K_I of ACG for adenosine is 3.0 mM, which is a ten-fold higher concentration than the K_I of 0.3 mM found in wild-type cells. This ten-fold lower sensitivity of ACG stimulation to adenosine could be related to the ten-fold higher concentrations of stimulus that is required to activate ACG relative to ACA.

Stimulation of ACG by dcAMP in vivo is strongly temperature dependent. The response as observed at 22°C, is absent when cells are stimulated at 0°C. Moreover, when a batch of ACG cells, that are stimulated at 22°C, is cooled instantaneously to 0°C, cAMP synthesis stops within 10 seconds. A strong temperature dependency has also been observed for the stimulation of GC, that shows a 13-fold reduction in the rate of receptor-stimulated cGMP formation *in vivo* at 0°C. In contrast, stimulation of ACA in wild-type cells is relatively temperature insensitive; the rate of receptor-stimulated cAMP formation in



Scheme 1. *Schematic representation of signal transduction in Dictyostelium discoideum.* cAR: cAMP receptor. FAR: Folic acid receptor. CRAC: cytosolic regulator of adenylyl cyclase GC: guanylyl cyclase ACA: adenylyl cyclase A, ACG: adenylyl cyclase G, α_2, α_4, β and γ: subunits of heterotrimeric G-protein. Hatched arrow: stimulus (folic acid or cAMP); dotted arrow: translocation of CRAC; solid arrow: activation pathway.

vivo is only 2.5-fold slower at 0°C than at 22°C (Van Haastert, 1987a). To determine the nature of the much stronger temperature-dependency of AGC relative to ACA, we measured the cAMP-forming activity of ACG at 0°C and at 22°C in the presence of Mg^{2+} and Mn^{2+}, and compared the results with those reported for ACA. Although the activity of ACG is very temperature dependent in the presence of Mg^{2+}, only a two-fold reduction in activity at 0°C is observed in the presence of Mn^{2+}. Moreover, these temperature dependencies are essentially identical to those of ACA activity *in vitro.* These results suggests that stimulation of ACA and ACG activates Mn^{2+}-dependent and Mg^{2+}-dependent activity, respectively. Alternatively, the factor that determines the temperature sensitivity in the dcAMP mediated activation of ACG is not the enzyme itself, but a component in the signal transduction pathway.

Folic acid is a chemoattractant for growing *Dictyostelium* cells. This compound binds to specific receptors on the cell surface and induces the activation of GC. Stimulation of ACA by folic acid could not be established. Previous experiments in wild-type cells yielded a minor cAMP response to folic acid, but this response was attributed to an artefact of the folic acid stimulus that amplifies the action of traces secreted cAMP, which in turn is produced by the basal active adenylyl cyclase (Devreotes, 1983). This conclusion is confirmed by the present observation that folic acid does not induce the activation of ACA in *car1⁻/car3⁻* cells. Interestingly, folic acid induces a large accumulation of cAMP levels in *aca⁻/ACG* cells. The artefact of folic acid on ACA regulation is ruled out in the case of ACG expressing cells, since stimulation by folic acid was also observed in *car1⁻/car3⁻/ACG* cells. Thus besides the structural resemblance that ACG shares with membrane-bound guanylyl cyclases, ACG and *Dictyostelium* GC both are stimulated by folic acid in vegetatively growing cells. Apparently, the transduction pathway from surface receptor to ACA contains a component that either is absent in vegetative cells or is not activated by folic acid receptors.

The association of cAMP to its receptors probably results in dissociation of the heterotrimeric G-protein G2, resulting in the release of the βγ complex from the Gα2 subunit. The membrane bound βγ-complex is thought to serve as a binding site for the cytosolic regulator of adenylyl cyclase CRAC, forming a membrane bound complex that subsequently activates ACA (Lilly & Devreotes, 1995). Cells lacking CRAC activity (*dagA*$^-$, *Synag7*) show no development and no cAMP mediated cAMP response (Insall *et al.*, 1994b). We observed that expression of ACG in a *crac*$^-$ mutant yields a cell line, that shows the normal activation of ACG activity upon stimulation of the cAMP receptor. Thus CRAC is not required for the activation of ACG. The role of a G-protein is more difficult to establish, because eight Gα-subunits have been identified, and deletion of the only known Gβ-subunit blocks development resulting in low levels of cAMP receptors (Pupillo *et al.*, 1989, Hadwiger *et al.*, 1991, Wu & Devreotes, 1991, Lilly & Devreotes, 1993). We could study the cAMP receptor mediated ACG stimulation in the absence of the heterotrimeric G-protein by over-expressing ACG together with cAR1 in *g*β$^-$ cells. In this mutant we observed a ten-fold increase of cAMP levels after stimulation with dcAMP. Also, when *g*β$^-$*/ACG* cells in vegetative stage are stimulated with folic acid, cAMP levels increase 2-3 fold, indicating that the heterotrimeric G-protein is not essential for excitation of ACG by dcAMP or folic acid. Folic acid receptors interact with the G-protein G4, and activation of the receptor will lead to the dissociation of the complex into Gα4 and the βγ-complex. Since *Dictyostelium* cells contain only one β-subunit, these experiments reveal that activation of ACG by receptors that interact with G-proteins is not mediated by those G-proteins. This conclusion is confirmed by experiments with ACG cell lysates and GTPγS, showing that GTPγS does not increase *in vitro* ACG activity. We conclude that stimulation of ACG activity by dcAMP proceeds via the cAR1 and/or cAR3 receptor. Unlike ACA, ACG does not depend on the translocation of CRAC to the membrane, nor is a G-protein involved in the stimulation of ACG. G-protein-independent signal transduction from serpentine receptors may seem unusual, but has recently been reported for the activation of G-box binding factor GBF and for the receptor stimulated Ca^{2+} entry in *Dictyostelium* (Schnitzler *et al.*, 1995; Milne *et al.*, 1995).

The regulation of ACA, ACG and GC, combined in scheme 1, leads to a proposal for signal transduction pathways. Activation of ACA is very complex, requiring G-proteins, CRAC and MAP kinase. Activation of GC is probably more direct; it does depend on G-proteins, but not on CRAC or MAP-kinase. Stimulation of ACG does not require any of these components. Whether this interaction between receptor and ACG is direct or mediated by an intracellular messenger remains unknown. A possibility is that receptor-stimulation of Ca^{2+} influx, which is G-protein-independent (Milne *et al.* 1995), in turn activates ACG. However, we observed that stimulation of ACG is not inhibited by excess extracellular EGTA, and Schaap *et al.* (1995) revealed that ACG *in vitro* activity is insensitive to Ca^{2+}, up to submillimolar concentrations. Another probability is that receptor stimulation leads to a pH change, which induces ACG. Schaap *et al.* demonstrated that ACG is sensitive for pH changes, with an optimum at pH 7.8. In conclusion, we state that ACG is a complete adenylyl cyclase, that combines characteristics of known adenylyl cyclases and guanylyl cyclases. The transduction pathway that links the cAMP receptor to ACG is unique for an adenylyl cyclase in that it does not require a G-protein.

ACKNOWLEDGEMENTS

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The role of guanylyl cyclase

Inhibition of receptor-stimulated guanylyl cyclase by intracellular calcium ions in *Dictyostelium* cells

Romi Valkema and Peter J.M. Van Haastert

Biochem. Biophys. Res. Comm. 186, 263-268

Chapter 4 - part I

ABSTRACT

In *Dictyostelium discoideum*, extracellular cAMP stimulates guanylyl cyclase and phospholipase C; the latter enzyme produces Ins(1,4,5)P_3, which releases Ca^{2+} from internal stores. The presented data indicate that intracellular Ca^{2+} ions inhibit guanylyl cyclase activity. 1) *In vitro*, Ca^{2+} inhibits guanylyl cyclase with IC_{50}=41 nM Ca^{2+} and Hill-coefficient of 2.1. 2) Extracellular Ca^{2+} does not affect basal cGMP levels of intact cells. In electro-permeabilized cells, however, cGMP levels are reduced by 85% within 45 s after addition of 10^{-6} M Ca^{2+} to the medium; halfmaximal reduction occurs at 200 nM extracellular Ca^{2+}. 3) Receptor-stimulated activation of guanylyl cyclase in electro-permeabilized cells is also inhibited by extracellular Ca^{2+} with half-maximal effect at 200 nM Ca^{2+}. 4) In several mutants an inverse correlation exists between receptor-stimulated Ins(1,4,5)P_3 production and cGMP formation. We conclude that receptor-stimulated cytosolic Ca^{2+} elevation is a negative regulator of receptor-stimulated guanylyl cyclase.

INTRODUCTION

The cellular slime mould *D. discoideum* uses extracellular cAMP for cell-cell communication during chemotaxis and differentiation (Devreotes, 1989; Schaap, 1986; Gerisch, 1987). cAMP binds to surface receptors, activates G-proteins and stimulates several second messenger systems, including adenylyl cyclase, guanylyl cyclase and phospholipase C. The produced cAMP is secreted in the medium where it can diffuse and activate neighboring cells. The produced cGMP remains largely intracellular, where it activates cGMP receptors or is degraded by a cGMP-stimulated cGMP-phosphodiesterase (Janssens *&* Van Haastert, 1987). The produced Ins(1,4,5)P_3 (Van Haastert *et al.*, 1989; Europe-Finner *et al.*, 1989) liberates Ca^{2+} ions from non-mitochondrial stores (Europe-Finner & Newell, 1986a).

The activation of adenylyl cyclase and phospholipase C are most likely mediated by GTP-binding regulatory proteins (Van Haastert *et al.*, 1991); the mechanism by which guanylyl cyclase is activated is less well understood. Earlier experiments with saponin treated cells revealed stimulation of guanylyl cyclase activity by Ca^{2+} ions (Europe-Finner, & Newell, 1985; Small *et al.*, 1986; Newell *et al.*, 1988). Recently, a Mg^{2+}-dependent guanylyl cyclase activity was identified in *D. discoideum* membranes that is strongly inhibited by Ca^{2+} (Janssens & De Jong. 1988; Janssens *et al.*, 1989) suggesting that *in vivo* guanylyl cyclase activity may be inhibited by Ca^{2+} ions rather than stimulated. We have analyzed the regulation of guanylyl cyclase by surface receptors and intracellular Ca^{2+} in electro-permeabilized cells and conclude that *in vivo* intracellular Ca^{2+} inhibits guanylyl cyclase in *D. discoideum*.

MATERIALS AND METHODS

Materials [^{3}H]cGMP and cGMP antiserum were obtained from Amersham.

Cells and culture conditions

D. discoideum cells (strain NC4) were grown on plates as described (Van Haastert *et al.*, 1989). Cells were harvested at the log-phase, washed three times with 10 mM KH_2PO_4/Na_2HPO_4, pH

6.5 (phosphate buffer), resuspended in this buffer to a density of 10^7 cells/ml, and starved for 4 hours.

Electro-permeabilization

Cells were washed three times in buffer A (20 mM HEPES, 1.5 mM $MgCl_2$, pH 7.0) resuspended in this buffer to a density of 10^8 cells/ml and electroporated by two 7 kV pulses discharged as described (Van Haastert *et al.*, 1989). Cells were immediately incubated in Ca^{2+}/EGTA buffers with 5.9 mM EGTA and different concentrations of $CaCl_2$, which were calculated using a K_D=1.85x10^8 for the Ca^{2+}/EGTA equilibrium constant at pH 7.0 (Bartfai, 1979).

cGMP response

Cells were stimulated with 0.1 µM cAMP and lysed at times indicated in the figure by the addition of 3.5% (vol/vol) perchloric acid. The cGMP content was measured in the neutralized extract by radioimmunoassay as described (Van Haastert & Van der Heijden, 1983).

Guanylyl cyclase assay (Janssens et al., 1989)

Cells were washed three times in 40 mM HEPES, pH 7.0, resuspended to 10^8 cells/ml in 40 mM HEPES, 3 mM $MgCl_2$, 50 µM GTPγS, 11.8 mM EGTA and different concentrations of $CaCl_2$, and lysed by rapid filtration through a 5 µm Nuclepore filter. At 30 s after lysis, the guanylyl cyclase reaction was started by mixing equal volumes of lysate and a mixture of 10 mM dithiothreitol and 0.6 mM GTP. The reaction was terminated with perchloric acid at 0, 30 and 60 s, and cGMP was measured in the neutralized extracts by radioimmunoassay (Janssens*et al.*, 1989).

RESULTS AND DISCUSSION

The activity of Mg^{2+}-dependent guanylyl cyclase in the presence of different Ca^{2+} concentrations is shown in figure 1. Enzyme activity was inhibited completely by micromolar Ca^{2+} concentrations; half-maximal inhibition was observed at about 41 nM. A Hill plot of these data yields a Hill coefficient of 2.1, indicating that inhibition of guanylyl cyclase by Ca^{2+} is positive cooperative. Guanylyl cyclase of rod outer segments is also inhibited by Ca^{2+} in a cooperative manner (Koch & Stryer, 1988).

Dictyostelium cells can be effectively permeabilized by electroporation (Van Haastert *et al.*, 1989; Schoen *et al.*, 1989). The conditions used produce very small holes which allow the transport of molecules smaller than about 300 Daltons. Thus, cells do not leak proteins or nucleotides such as ATP or GTP (Van Haastert *et al.*, 1989; Van Duijn *et al.*, 1990). Electro-permeabilized cells in EGTA show a strong increase of cGMP levels upon stimulation with cAMP (fig. 2). Addition of 10^{-6} M Ca^{2+} to electro-permeabilized cells leads to a decrease of basal cGMP levels, and subsequent cAMP stimulation induces only a small cGMP response. Basal and cAMP-stimulated cGMP levels were measured at different extracellular Ca^{2+} concentrations in electro-permeabilized cells, showing that both are equally inhibited with IC_{50} = 200 nM Ca^{2+} (fig. 3). Extracellular Ca^{2+} had no effect on basal cGMP levels of intact *D. discoideum* cells (fig. 3), suggesting that the inhibition by Ca^{2+} in electro-permeabilized cells was due to changes of the intracellular Ca^{2+}

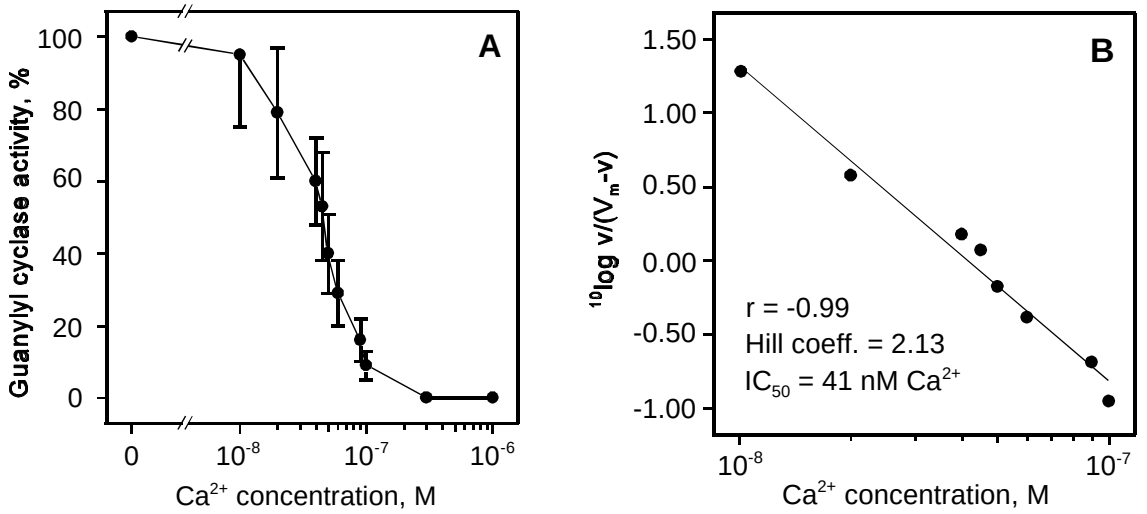

Figure 1. *The regulation of guanylyl cyclase by Ca^{2+} in vitro.*
A, Guanylyl cyclase activity was measured in a cell-free preparation at different free Ca^{2+} concentrations; half maximal inhibition occurred at 41 nM Ca^{2+}. B, Hill plot of the same data; the Hill coefficient is n=2.1.

concentration.

It has been proven difficult to measure cytosolic Ca^{2+} concentrations in *Dictyostelium* cells. Cytosolic Ca^{2+} concentrations are likely to be regulated partly by Ins(1,4,5)P_3. To establish a possible regulation of guanylyl cyclase by Ca^{2+} *in vivo*, we have collected data on receptor-mediated formation of both cGMP and Ins(1,4,5)P_3 in intact cells for a variety of mutants (table I). The cAMP mediated activation of phospholipase C was lost in mutant *fgd*C and the cGMP response was slightly larger than in wild-type cells (Bominaar *et al.*, 1991). Transformants overexpressing a mutated ras gene (Dd-RAS-THR[12]) showed an increased formation of Ins(1,4,5)P_3 (Europe-Finner *et al.*, 1988) due to the enhanced conversion of phosphatidyl-inositol to phosphatidylinositolphosphate (Van der Kaay *et al.*, 1990). This effect was associated with an increased activity of a protein kinase C-like enzyme (Ludérus *et al.*, 1988). Thus, it is expected that in mutant Dd-RAS-THR[12] both Ins(1,4,5)P_3, Ca^{2+} and PKC activities are increased. The cGMP response is diminished in this transformant (Van Haastert *et al.*, 1987b). Finally, in wild-type cells the partial antagonist 8-p-chloro-phenylthioadenosine 3',5'-cyclic monophosphate induced a decrease of Ins(1,4,5)P_3 levels, whereas a very strong cGMP response was induced (Peters *et al.*, 1991).

The experiments with electroporated cells in EGTA and previous data of experiments with mutant cells clearly demonstrate that receptor-mediated cGMP formation can occur in the absence of receptor-mediated stimulation of phospholipase C as well as in the

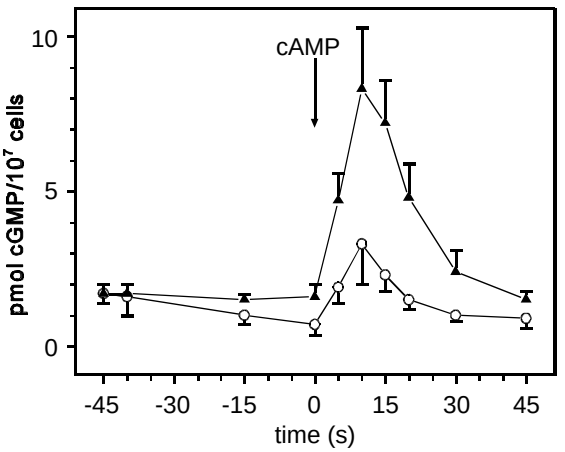

Figure 2. *Ca^{2+} regulation of the cAMP-induced cGMP response in permeabilized cells*.
Cells were electro-permeabilized and preincubated for 45 s with 5.9 mM EGTA (△) or 5.9 mM EGTA with 1 µM free Ca^{2+} (o). Cells were then stimulated at t=0 with 0.1 µM cAMP, lysed at the times indicated and cGMP levels were measured.

absence of elevated intracellular Ca^{2+} concentrations. These results confirm experiments on the effect of Ca^{2+} on guanylyl cyclase activity *in vitro*, showing that this bivalent cation is a potent inhibitor of enzyme activity. In contrast to previous results with saponin treated cells (Europe-Finner & Newell, 1985; Small *et al.*,, 1986; Newell *et al.*, 1988), the present results imply that *in vivo* intracellular Ca^{2+} inhibits guanylyl cyclase.

The activity of guanylyl cyclase in membranes without Ca^{2+} and the rate of cGMP accumulation in intact cells upon stimulation with cAMP are nearly identical (both 40-60 pmol/min per equivalent of 10^7 cells). This may suggest that in unstimulated cells guanylyl cyclase is inhibited by Ca^{2+} and that cAMP stimulation of enzyme activity is mediated by the loss of this inhibition. We could not find evidence for this hypothesis, because basal and cAMP-stimulated cGMP levels show the same sensitivity for Ca^{2+} (fig. 3).

In aggregation competent cells, guanylyl cyclase is activated by extracellular cAMP, whereas folic acid stimulates the enzyme in growing cells. Folic acid and cAMP are detected by different surface receptors, but share a common guanylyl cyclase (Van Haastert, 1983a). The activation of guanylyl cyclase by cAMP is probably mediated by the receptor cAR1, because the cyclic nucleotide specificity for binding to cAR1 is identical to the specificity for guanylyl cyclase activation (Van Haastert & Kien, 1983; Johnson *et al.*, 1992) and cAMP-stimulation of guanylyl cyclase is lost in cells with reduced expression of cAR1 (Sun *et al.*, 1990). Besides cAR1 and guanylyl cyclase, an additional component is required for stimulation by cAMP, because cAMP cannot stimulate guanylyl cyclase in cells that overexpress cAR1 during growth; these cells do express

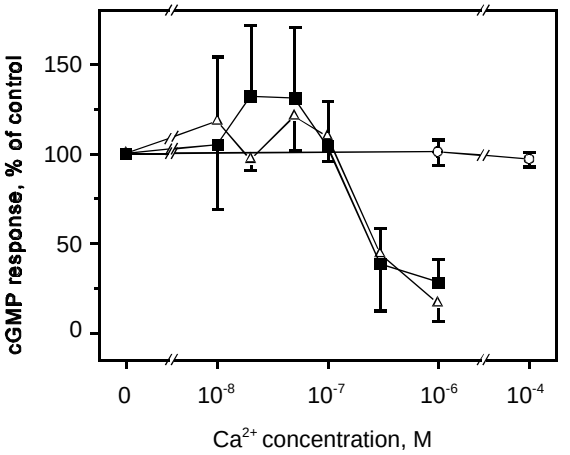

Figure 3. *The regulation of basal and stimulated cGMP levels by Ca^{2+}*.
Electro-permeabilized cells were incubated for 45 s at different free Ca^{2+} concentrations and stimulated with 0.1 µM cAMP. cGMP levels were measured just before (■) and 10 s after stimulation (△). Basal cGMP levels were also measured in non-permeabilized cells (o). Data are presented as means and standard deviations relative to the control without Ca^{2+}. The control levels in pmol/10^7 cells were: 0.67 ± 0.26 pmol for (■), 10.0 ± 1.9 pmol for (△), and 0.71 ± 0.13 pmol for (o).

guanylyl cyclase which can be activated by folic acid (Johnson *et al.*, 1991 and unpublished results). The missing component could be a G-protein, as

Table I. *The regulation of cGMP and Ins(1,4,5)P_3 in D. discoideum cells*

Condition	cGMP	Ins(1,4,5)P_3	Ref
Response of mutant *fgd*C to cAMP	increased	reduced	[1]
Basal levels in mutant Dd-RAS-THR12	normal	increased	[2],[3]
Response of wild-type cells to 8-CPT-cAMP	increased	reduced	[4]

[1] Bominaar *et al.*, 1991; [2]Van der Kaay *et al.*, 1990; [3] Van Haastert *et al.*, 1987b; [4] Peters *et al.*, 1991.

mutant *fgd*A with a defective Gα2- subunit fails to show cAMP-simulated guanylyl cyclase, whereas stimulation by folic acid is unaltered (Kesbeke *et al.*, 1988). These observations suggest that the sensory transduction pathways from surface receptor to guanylyl cyclase may include different receptors for cAMP and folic acid, different G-proteins and a common guanylyl cyclase. In this scheme Ca^{2+} is a negative regulator of guanylyl cyclase activity *per se*, but is not involved in the activation mechanism of the enzyme.

The negative regulation of guanylyl cyclase by Ca^{2+} ions has also been described for the enzyme from bovine retinal rods (Dizhoor *et al.*, 1991), where the protein recoverin mediates this inhibition. Possibly the guanylyl cyclase activity in *Dictyostelium* is regulated by a similar protein, especially since the inhibition by Ca^{2+} shows the same sensitivity and cooperativity for Ca^{2+} with both enzymes. In *Paramecium*, however, the opposite is found: Ca^{2+} ions stimulate guanylyl cyclase activity (Klumpp & Schultz, 1982).

In conclusion we have demonstrated that Ins(1,4,5)P_3-mediated Ca^{2+} release is a negative regulator of guanylyl cyclase activity. This suggests that Ca^{2+} and cGMP may have partially antagonistic functions in *D. discoideum*. Guanylyl cyclase and phospholipase C are activated most likely by the same surface receptor. The inhibition of guanylyl cyclase by Ca^{2+} may induce or amplify existing intracellular gradients of cGMP and Ca^{2+}. Therefore, inhibition of guanylyl cyclase by Ca^{2+} may help the cell to orient effectively in chemotactic gradients of extracellular cAMP.

ACKNOWLEDGMENTS

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The role of guanylyl cyclase

A model for cAMP-mediated cGMP response in *Dictyostelium discoideum*

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Chapter 4 - part II

ABSTRACT

In *Dictyostelium discoideum* extracellular cAMP, as shown by previous studies, induces a transient accumulation of intracellular cGMP, which peaks at 10 s and recovers basal levels at 30 s after stimulation, even with persistent cAMP stimulation. Additional investigations have shown that the cAMP-mediated cGMP response is build up from surface cAMP receptor-mediated activation of guanylyl cyclase and hydrolysis of cGMP by phosphodiesterase. The regulation of these activities was measured in detail on a seconds time-scale, demonstrating complex adaptation of the receptor, allosteric activation of cGMP-phosphodiesterase by cGMP, and potent inhibition of guanylyl cyclase by Ca^{2+}. In this paper we present a computer model that combines all experimental data on the cGMP response. The model is used to investigate the contribution of each structural and regulatory component in the final cGMP response.

Four models for the activation and adaptation of the receptor are compared to experimental observations. Only one model describes the magnitude and kinetics of the response accurately. The effect of Ca^{2+} on the cGMP response is simulated by changing the Ca^{2+} concentrations outside the cell (Ca^{2+} influx), in stores (IP_3-mediated release) and changing phospholipase C activity. The simulations show that Ca^{2+} mainly determines the magnitude of the cGMP accumulation; simulations are in good agreement with experiments on the effect of Ca^{2+} in electropermeabilized cells. Finally, when cGMP-phosphodiesterase activity is deleted from the model, the simulated cGMP response is elevated and prolonged, which is in close agreement with the experimental observations in mutant *stm*F that lacks this enzyme activity. We conclude that the computer model provides a good description of the observed response, suggesting that the main structural and regulatory components have been identified.

INTRODUCTION

The slime mold *Dictyostelium discoideum* lives in the soil where it feeds on bacteria. Upon food depletion the unicellular amoebae organize in a multicellular slug, in which differentiation occurs. The cells in the anterior part develop into stalk cells, whereas the cells in the posterior part will become spores (Schaap & Wang, 1986). The development of *Dictyostelium* is triggered by cAMP, which is secreted by the amoebae upon starvation (Konijn, 1972). Neighboring cells are capable of responding to the cAMP gradient by means of cAMP receptors in the cell membrane (Malchow & Gerisch, 1974; Green & Newell, 1975; Henderson, 1975; Mato & Konijn, 1975). Stimulation of these receptors triggers a cascade of reactions, which finally results in cell movement towards the increasing concentration of cAMP (Gerisch *et al.,* 1975).

Upon stimulation of the cAMP receptor the intracellular enzymes guanylyl cyclase and phospholipase C are rapidly activated (Mato & Malchow, 1978; Europe-Finner & Newell, 1987). Consequently the concentrations of cGMP, inositol 1,4,5-trisphosphate (IP_3) and Ca^{2+} increase, myosin is phosphorylated and actin polymerizes, eventually resulting in enhanced and directed cell motility (Malchow *et al.,* 1981; Europe-Finner & Newell, 1986a; McRobbie & Newell, 1984; Europe-Finner & Newell, 1986b; Liu & Newell, 1988). *Dictyostelium* exhibits chemotaxis towards different chemoattractants like cAMP and folic acid (Pan *et al.*, 1972; Konijn *et al.*, 1967). The

role of cGMP in chemotaxis has been emphasized in *stm*F, a mutant which, due to the absence of cGMP-specific phosphodiesterase, has an increased cGMP response and shows prolonged chemotactic movement towards cAMP and folic acid (Ross & Newell, 1981; Van Haastert *et al.*, 1982). The conclusion that cGMP is involved in chemotaxis was recently confirmed in experiments with mutant KI8. This mutant, with strongly reduced guanylyl cyclase activity, shows no chemotaxis to either cAMP or folic acid (Kuwayama *et al.*, 1993).

cGMP levels start to increase at about one second after stimulation of the cells with cAMP, peak levels are achieved ten seconds later (Van Haastert, 1987a). Subsequently the concentration of cGMP declines to reach basal levels at approximately 30 seconds (Mato *et al.*, 1977b). Several experiments suggest that the receptor-mediated cGMP response is regulated by complex mechanisms (Van Haastert & Van der Heijden, 1983). Although the peak values of the cGMP response depend on the stimulus concentration, the kinetics of the response is essentially independent with respect to the cAMP concentration. Extracellular cAMP is degraded by phosphodiesterase activity in the medium (Chang, 1968; Panbacker & Bravard, 1972; Malchow *et al.*, 1972). The magnitude and kinetics of the cGMP response remain the same whether the cAMP stimulus is present for only 3 seconds or is not degraded at all (Van Haastert & Van der Heyden, 1983). Finally, when cells are stimulated twice at 30 s interval, they respond only to the second stimulus if the concentration is higher than that of the first stimulus (Van Haastert, 1983a). These experiments indicate that the receptor-mediated cGMP response is regulated by an adaptation mechanism.

Biochemically, the cGMP response is controlled at two points: synthesis by guanylyl cyclase and degradation by phosphodiesterase. Guanylyl cyclase is stimulated by the receptor (Mato & Malchow, 1978). Previous studies have indicated that adaptation of the cGMP response occurs upstream of guanylyl cyclase (Van Haastert, 1983a), presumably at the receptor or at the Gα2-protein (Okaichi *et al.* 1992). Detailed kinetic studies of cAMP binding to *Dictyostelium discoideum* cells suggest that a subpopulation of surface receptors is involved in the activation of guanylyl cyclase and that adaptation is associated at the interconversions between active and inactive receptor forms (Van Haastert *et al.*, 1986). Guanylyl cyclase activity is inhibited by Ca^{2+} ions (Janssens *et al.*, 1989; Valkema & Van Haastert, 1992), suggesting that the cGMP response is regulated by receptor-stimulated Ca^{2+} uptake as well as by phospholipase C and IP_3 via the release of Ca^{2+} from internal stores (Van Haastert *et al.*, 1989; Streb *et al.*, 1983; Bumann *et al.*, 1984). Two classes of phosphodiesterases participate in intracellular cGMP degradation. Intracellular cGMP is degraded mainly by a cGMP-specific enzyme that is stimulated by cGMP at low concentrations. About 20% of intracellular cGMP is degraded by a less specific enzyme (Van Haastert *et al.*, 1983). In summary, the cGMP response is controlled by a cGMP-stimulated phosphodiesterase and Ca^{2+}-inhibited guanylyl cyclase, that is stimulated by a surface cAMP receptor that is subjective to adaptation. The contribution of each of these regulatory components to the final cGMP response is essentially unknown, and can not easily be determined in

experiments.

The kinetic values of nearly all biochemical reactions described above have been determined in previous experiments on the time scale of the cGMP response (seconds). In order to determine the contribution of receptor adaptation, Ca^{2+} inhibition of guanylyl cyclase and cGMP-stimulated phosphodiesterase activity to the final cGMP-response we translated the observed reactions and kinetic values of all enzymes into a model. This model consists of five differential equations, which describe the activated cAMP receptor, the changes in the concentration of cGMP, IP_3 and Ca^{2+}, and the activity of cGMP-specific phosphodiesterase, respectively. Different adaptation mechanisms were investigated, revealing that a specific adaptation regime is essential to describe the observed transient response. The model predicts that adaptation determines the appearance of the cGMP response curve, Ca^{2+} inhibition of guanylyl cyclase determines the magnitude of the response, whereas the cGMP stimulated phosphodiesterase determines the duration of the response. Finally the cGMP response in two signal transduction mutants was simulated by deleting phosphodiesterase activity and phospholipase C activity from the model; the predictions were similar to experimental data. We conclude that the model describes experimental data, suggesting that the main structural and regulatory elements of cGMP metabolism are included into the model.

MATERIALS AND METHODS

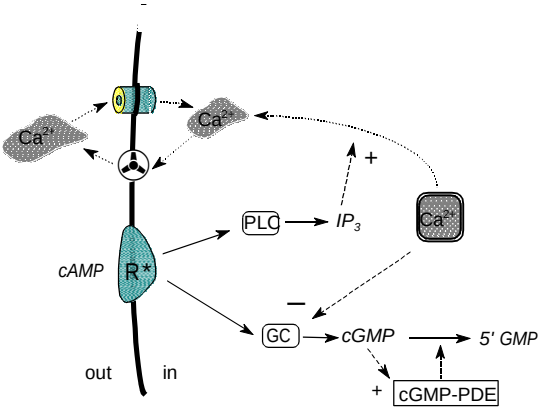


Figure 1. *Schematic representation of intramolecular interactions contributing to the cGMP response in Dictyostelium discoideum*. R^*, stimulated cAMP receptor; GC, guanylyl cyclase; PLC, phospholipase C; cGMP-PDE, cGMP-stimulated cGMP-specific phosphodiesterase.

The relations between the different components that determine intracellular cGMP levels are presented in figure 1. cGMP is degraded mainly by a cGMP stimulated phosphodiesterase. Guanylyl cyclase produces cGMP; the enzyme is stimulated by an activated receptor (denoted by R^*) and is inhibited by intracellular Ca^{2+} levels. The concentration of Ca^{2+} is controlled by receptor stimulated IP_3 levels and by receptor stimulated Ca^{2+} uptake. The change of cGMP concentration is given by equation 1, where *f SYN* is the synthesis of cGMP and *f DEG* is its degradation.

$$\frac{d[cGMP]}{dt} = f\,SYN - f\,DEG \qquad (1)$$

cGMP synthesis

The enzyme guanylyl cyclase hydrolyses GTP to cGMP. In *Dictyostelium* this enzyme is likely a membrane associated protein (Mato & Malchow, 1978; Janssens *et al.*, 1989). The rate of cGMP synthesis

is given by

$$f\,SYN = [\,1 - \eta \frac{[Ca^{2+}]^n}{[Ca^{2+}]^n + [K_I]^n}][\delta + \varepsilon R^*] \qquad \textbf{(1a)}$$

where η is the fraction of guanylyl cyclase that is sensitive to Ca^{2+} inhibition. *In vitro* all guanylyl cyclase activity is sensitive to Ca^{2+} inhibition (η=1); in electro-permeabilized cells approximately 20% of guanylyl cyclase activity remains active in the presence of 1 mM Ca^{2+} (η=0.8) (Van Haastert, unpublished results). K_I is the concentration of Ca^{2+} that induces half maximal inhibition (K_I=200 nM); inhibition of guanylyl cyclase by Ca^{2+} is a cooperative process with a Hill coefficient n=2.3 (Janssens *et al.*, 1989; Valkema & Van Haastert, 1992). δ and ε represent the enzyme activity of guanylyl cyclase in basal and receptor-activated state, respectively. The values of these constants have been measured and are given in table I.

cGMP degradation

The hydrolysis of cGMP to 5′-GMP is performed by two cyclic nucleotide phosphodiesterase activities: a small phosphodiesterase activity hydrolyzing cAMP and cGMP at approximately the same rate, and a large activity specific for cGMP (Chang, 1968; Van Haastert *et al.,* 1983). cGMP stimulates the latter enzyme about three-fold by decreasing the K_m of the enzyme at an unaltered V_{max} (Bulgakow & Van Haastert, 1983). The activity of phosphodiesterases in the model is designated by the following equation:

$$f\,DEG = (1 -)V_G \frac{[cGMP]}{[cGMP] + K_M^L} + V_G \frac{[cGMP]}{[cGMP] + K_M^H} + V_A \frac{[cGMP]}{[cGMP] + K_M^A} \qquad \textbf{(1b)}$$

In this equation V_G and V_A are the V_{max} of the cGMP-specific and the non-specific enzyme, respectively; K_M^L and K_M^H are the Michaelis-Menten constants of the cGMP-specific enzyme in the low and high active form, respectively. K_M^A is the Michaelis-Menten constant of the non-specific phosphodiesterase. is the fraction of the cGMP specific enzyme in the activated state, which is given by

$$\frac{d}{dt} = k\,[cGMP](1 -) - k_- \qquad \textbf{(1c)}$$

k and k₋ are allosteric rate constants of activation and deactivation of the cGMP specific phosphodiesterase. Detailed studies of cGMP degradation have provided the values of all constants (Van Haastert & Van Lookeren Campagne, 1984), which are given in table I.

Regulation of intracellular Ca^{2+} levels

Calcium ions inhibit guanylyl cyclase activity. Stimulation of the cAMP receptor induces influx of extracellular Ca^{2+} (Bumann *et al.*, 1984) and activates phospholipase C whereby phosphatidylinositol-bisphosphate is hydrolyzed to IP_3 and diacylglycerol. IP_3 liberates Ca^{2+} from non-mitochondrial internal stores (Europe-Finner & Newell, 1986a). The IP_3 concentration is given by

$$\frac{d[IP_3]}{dt} = \alpha + R^* - [IP_3] \qquad \textbf{(2a)}$$

where α and ß are the basal and receptor stimulated activity of phospholipase C respectively (Bominaar *et al.,* 1993), and is the first order rate constant of IP_3 degradation (Van Lookeren Campagne *et al.*, 1988).

The Ca^{2+} concentration of the cytosol is described by:

$$\frac{d[Ca^{2+}]_{cyt}}{dt} = \frac{V_c^L [Ca^{2+}]_{out}}{K_m^{cL} + [Ca^{2+}]_{out}} + \frac{V_c^H [Ca^{2+}]_{out}}{K_m^{cH} + [Ca^{2+}]_{out}} R^* + [C + D \frac{[IP_3]^M}{[IP_3]^M + q^M}][Ca^{2+}]_{store} - E [Ca^{2+}]_{cyt} - F [Ca^{2+}]_{cyt} \quad \textbf{(2b)}$$

The first part of the equation denotes the plasma membrane channels that transport Ca^{2+} to the cytosol, which follow Michaelis Menten kinetics. Activation of the receptor alters both the V_{max} and the $_mK$ of the transport. The values of these constants have been measured (Millne & Coukell, 1991) and are presented in table I.

The second part of the equation represents the IP_3-mediated release of Ca^{2+} from non-mitochondrial stores (Europe-Finner & Newell, 1986a). Details of this reaction have not been determined in *Dictyostelium*; we assume values of reaction constants, which have been measured in mammalian cells (Champeil *et al.,* 1989; Streb *et al.*, 1983). The Ca^{2+} concentration in the IP_3-sensitive store is assumed to be 1 mM. The release of Ca^{2+} from the store by IP_3 is assumed to occur in a co-operative way, with a Hill coefficient M=2 and a half maximal activity at q=1.10 µM.

The third part of the equation denotes the Ca^{2+} pump activity E back to the extracellular medium, and F back to the intracellular store. In unstimulated cells the influx of Ca^{2+} from the extracellular medium equals the efflux :

$$\frac{V_c [Ca^{2+}]_{out}}{K_m^c + [Ca^{2+}]_{out}} = E [Ca^{2+}]_{cyt} \quad \textbf{(2c)}$$

Assuming a basal cytosolic Ca^{2+} concentration of $5x10^{-8}$M (Abe, 1988) and an extracellular Ca^{2+} concentration of 10 µM (Bumann *et al.*, 1984) implies E= 6 s^{-1}. In unstimulated cells the efflux from the intracellular Ca^{2+} store equals the flux of Ca^{2+} ions pumped back in this store yielding F= 6 s^{-1}.

Activation and adaptation of the surface cAMP receptor

Binding of cAMP to the surface receptor induces the accumulation of cGMP levels. The response is transient with maximal cGMP levels at 10s and a recovery of basal cGMP levels after 30s, even during persistent stimulation with cAMP. Partial desensitization could be provided by the Ca^{2+}-mediated inhibition of guanylyl cyclase and cGMP-stimulation of cGMP-phosphodiesterase; this will be investigated in a model called simple adaptation. Several experiments suggest that desensitization is mediated by adaptation occurring at the level of the cAMP surface receptor (Van Haastert & Van der Heijden, 1983; Van Haastert, 1987c). Therefore alternative models were analyzed for different adaptation regimes.

Simple adaptation

The binding of cAMP to the receptor is a simple bimolecular reaction, and the occupied receptor remains in the activated state (scheme I). Adaptation

does not occur at the receptor, but intracellularly at the level of cGMP synthesis or degradation. The differential equation for the occupied activated receptor R^* is:

$$\frac{dR^*L}{dt} = k_1[cAMP](1 - R^*L) - k_{-1}R^*L$$

(3a)

Linear adaptation

This model introduces the adapted occupied receptor state R^DL, which is formed from the activated occupied receptor R^* (scheme II). The differential equation for the activated occupied receptor R^* and for the occupied receptor R^DL are:

$$\frac{dR^*L}{dt} = k_1[cAMP](1 - R^*L - R^DL) - k_{-1}R^*L - k_2R^*L + k_{-2}R^DL$$

$$\frac{dR^DL}{dt} = k_2R^*L - k_{-2}R^DL$$

(3b)

Box-model

The receptor box model is based on a study on the activation of adenylyl cyclase in *Dictyostelium* (Knox *et al.*, 1986, Goldbeter & Koshland, 1982). The model assumes two interconvertible forms of the receptor R^S and R^D, respectively. Each form of the receptor can associate with the ligand cAMP, yielding R^SL and R^DL, respectively (scheme III). All four receptor states possess a specific activity a_x. The total receptor activity R^* is denoted as follows:

$$R^* = a_1R^S + a_2R^SL + a_3R^DL + a_4R^D$$

(3c)

Experimental data indicate that the association of ligand to the receptor is much faster than the interconversion between the receptor forms, thus:

$$\frac{dR^D}{dt} = k_3R^S - k_{-3}R^D$$

$$\frac{dR^DL}{dt} = \frac{k_4R^S[cAMP]}{K_R} - \frac{k_{-4}R^D[cAMP]}{K_D}$$

(3d)

Cycle-model

The Cycle-model describes the adaptation process as a series of sequential interconversions of receptor forms. This model was based on kinetic studies of the interaction between cAMP and a subpopulation of receptors that are supposed to be involved in the activation of guanylyl cyclase (Van Haastert et al., 1986). cAMP binds reversibly to the receptor, yielding RL. This receptor form converts with the rate k_x to the activated state of the receptor R^*L. k_x is not a constant, but declines with time according $k_x=0.22e^{-0.17t}$ s^{-1}. The active receptor R^*L then converts to a desensitized state R^DL with a rate constant $k_y=0.17$ s^{-1}. R^DL slowly converts back to the inactive receptor RL with $K_z = 7.3 \times 10^{-3}$ s^{-1} (Van Haastert et al., 1986; Van Haastert, 1987c).

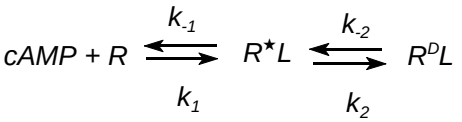
The differential equations for the different receptor forms are:

$$\frac{dR^*L}{dt} = k_xRL - k_yR^*L$$

$$\frac{dR^DL}{dt} = k_yR^*L - k_zR^DL$$

$$\frac{dRL}{dt} = k_1[cAMP](1 - RL - R^*L - R^DL) - k_{-1}RL - k_xRL$$

(3e)



scheme 2

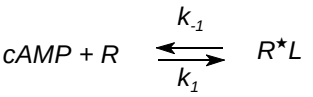
RESULTS

Adaptation of the model

A typical cGMP response of starved Dictyostelium cells upon cAMP stimulation is shown in figure 2A. After a delay of about one second the cellular cGMP concentration increases and reaches a peak level at 10 seconds; basal conditions are recovered at 30 seconds after the addition of the stimulus. In vivo measurements show that the magnitude of the response increases with increasing concentrations of the cAMP stimulus, whereas the kinetics of the cGMP response is essentially independent of the stimulus concentration. Furthermore, cGMP levels always return to pre-stimulus concentrations at about 30 s after cAMP stimulation, independent of the dynamics of the stimulus (rapid or no degradation of cAMP; Van Haastert & Van der Heyden, 1983). In this section four adaptation models are investigated on the kinetics of the cGMP response. Simulations were performed for 50 s with constant cAMP concentrations at 10^{-8} M, 10^{-7} M and 10^{-6} M.

The Simple adaptation model

Previous experiments revealed inhibition of guanylyl cyclase by Ca^{2+} and stimulation of cGMP-phosphodiesterase by cGMP. The simple adaptation model investigates whether these negative control elements are sufficient to explain the observed desensitization of the cGMP response. This model predicts (fig. 2B) that the cGMP concentration will reach a peak at 10 s after stimulation; the cAMP dose dependency of the cGMP response also agrees with experimental observations. Furthermore, cGMP levels do decline after 10 s of stimulation. However, this decline is only about 15% of the cGMP peak at 10 s, which is far less than observed experimentally. Thus, although the cGMP response in this simple adaptation model already shows



scheme 1

some adaptation characteristics, cGMP levels do not recover basal levels according to experimental observations. We conclude that the simple model shows poor desensitization, indicating that the negative regulation of guanylyl cyclase by Ca^{2+} and the positive regulation of phosphodiesterase by cGMP are insufficient to obtain complete desensitization. In the subsequent models adaptation will be included at the level of the receptor.

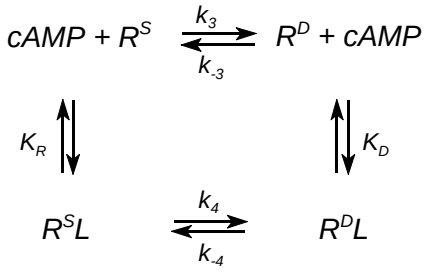
The Linear-adaptation model

In this model the activated receptor R^* converts to a desensitized form R^D. The Linear-adaptation model predicts a cGMP-peak between 6 and 9 seconds after stimulation (fig. 2C). The response is cAMP-dose dependent. However, the response does not adapt completely: at 50 s after stimulation the cGMP concentration is still 15% above basal level. Furthermore, the model predicts

specific kinetics of the response, which have not been observed experimentally: the response and recovery to basal levels is fast at high concentrations of cAMP, and slow at low concentrations.

The Box-adaptation model

The model is based on experimental observations of cAMP-binding to surface receptors that are supposed to interact with adenylyl cyclase in *Dictyostelium* (Knox *et al.,* 1986). cAMP can interact with two interconvertible forms of the receptor; each of the occupied and unoccupied receptor forms possesses different activity. Simulation of the box-adaptation model reveals complete adaptation of the cGMP response at each stimulus concentration (fig. 2D). The model predicts that the kinetics of the cGMP response alters at different concentrations of the cAMP stimulus: at higher stimulus concentrations the response increases and returns to basal levels faster than at lower stimulus concentrations. This has not been observed for the cAMP-stimulation of guanylyl cyclase (see figure 2A).



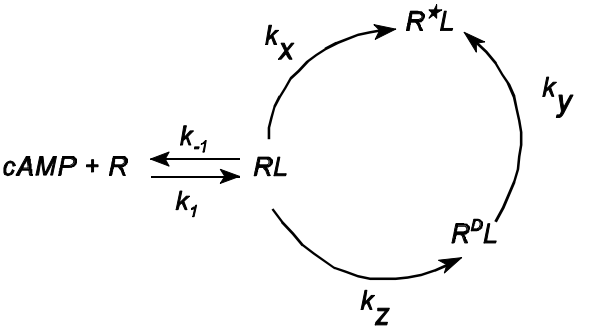
scheme 3

Furthermore, the model predicts that the rate of cGMP increase is maximal immediately after cAMP addition (fig. 2D), whereas experimental observations reveal a 1 s lag period between cAMP addition and the increase of cGMP levels (see figure 2A, inset) (Van Haastert, 1987a). Although the box-adaptation model predicts perfect adaptation, several properties of the predicted response are not in good agreement with experimental observations for the cGMP response.

The Cycle-adaptation model

This model is based on experimental observations on the binding of cAMP to a subpopulation of surface cAMP receptors that are supposed to be involved in the activation of guanylyl cyclase (Van Haastert, 1987c). cAMP binds reversibly to the inactive receptor R, which sequentially converts to an active form R^* and to a desensitized form R^D, which slowly recovers to the inactive receptor R. The rate constants of these interconversions have been measured (Van Haastert, 1987c). The Cycle-model predicts a response, which shows perfect adaptation (fig. 2E). Furthermore, the kinetics of the response is independent of the cAMP stimulus concentration. Finally, the predicted response exhibits a short delay before the cGMP concentration rises rapidly to a peak at 8-10 s; basal levels are recovered at 30 s after stimulation.

Considering these data we conclude that the cycle-adaptation model fits best with experimental observations. Therefore this cycle-model was used to perform the following experiments on the role of cGMP



scheme 4

phosphodiesterase and intracellular Ca^{2+}.

cGMP degradation

Intracellular cGMP is hydrolyzed by two cyclic nucleotide phosphodiesterases: a non-specific phosphodiesterase with low activity and a cGMP-specific cGMP-phosphodiesterase with high activity (Van Haastert *et al.*, 1983). The latter enzyme is stimulated about three-fold by cGMP with a half-time of about 20 s (Van Haastert & Van Lookeren Campagne, 1984). The role of the cGMP-specific phosphodiesterase for the receptor-stimulated cGMP response was studied by simulating the absence of cGMP specific enzyme activity (V_G = 0), or by simulating an enzyme that cannot be activated by cGMP (k = 0). The results (fig. 3A) reveal in both cases that the cGMP response is increased and prolonged. When cGMP cannot activate the enzyme, cGMP peak levels are increased with a factor 1.7 relative to the response with normal phosphodiesterase; the cGMP peak is reached at 14 seconds and basal levels are recovered after 50 s. When the enzyme is absent, the cGMP response is enhanced with a factor of 3.5 relative to the control response; the peak is reached after 22 s, and basal levels do not recover within 100 s.

A *Dictyostelium* mutant *stm*F has been isolated that lacks the cGMP-specific phosphodiesterase (Ross & Newell, 1981; Van Haastert *et al.*, 1982). The cAMP-mediated cGMP response in this mutant (fig. 3B) closely resembles the calculated cGMP levels: a prolonged and increased response, with recovery of the basal cGMP levels at 100-120 s after stimulation.

Intracellular Ca^{2+} levels

Guanylyl cyclase in *Dictyostelium* is strongly inhibited by intracellular Ca^{2+} ions with half-maximal inhibition at 200 nM and a Hill coefficient of 2.3 (Valkema & Van Haastert, 1992). Cytosolic Ca^{2+} concentrations are regulated in a complex manner that are not completely understood in *Dictyostelium*. In the model we have incorporated experimental data on the cAMP surface receptor-mediated uptake of Ca^{2+}, and on the release of Ca^{2+} from intracellular stores by IP_3 that is produced by receptor stimulated phospholipase C. The role of Ca^{2+} was investigated by simulating the absence of phospholipase C activity and modifying Ca^{2+} concentrations in the extracellular medium or in the intracellular stores.

Removal of phospholipase C activity from the model predicts a cGMP response that is only 1.2-fold higher than the response of cells that do possess phospholipase C activity (fig. 4A).

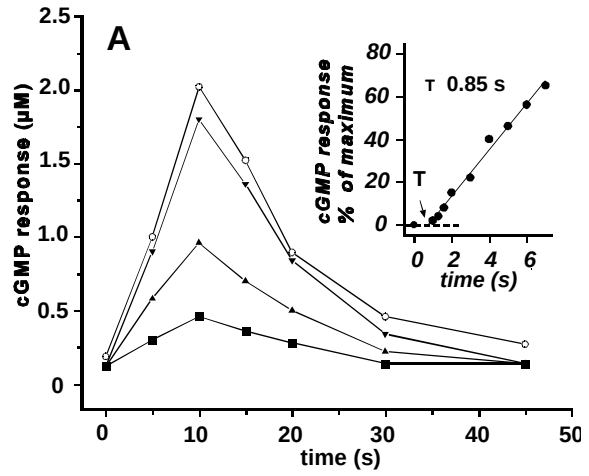
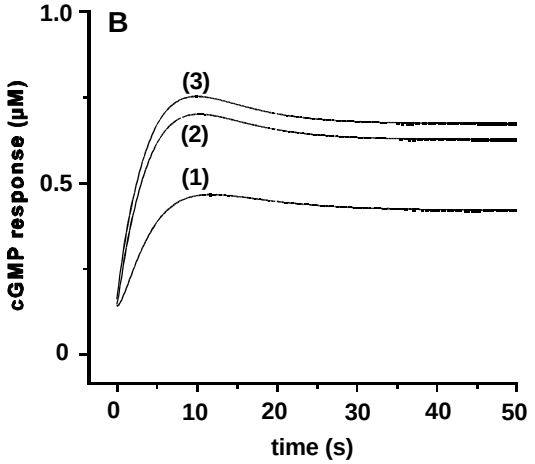
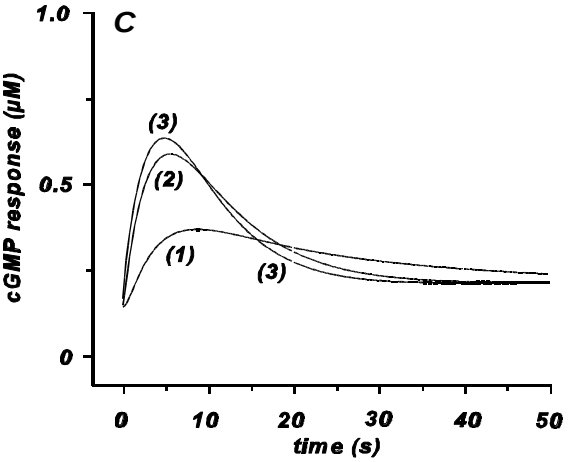
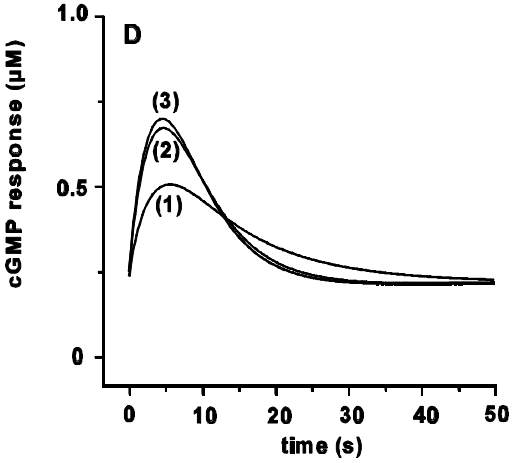
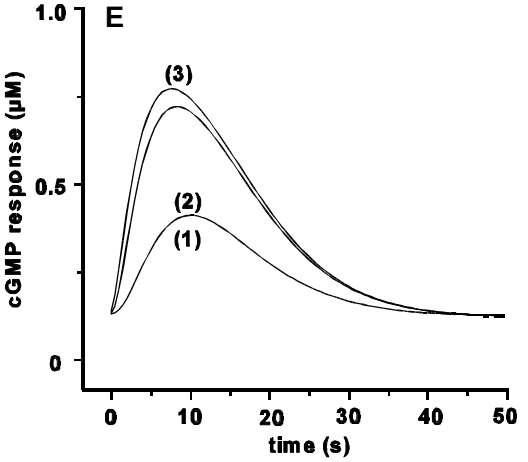


Figure 2. *Time course of cGMP formation upon stimulation with different cAMP concentrations*. Panel A: experimental observations, cAMP = $2x10^{-9}$M (■), 10^{-8} M (▲), 10^{-7} M (▼), 10^{-6} M (○) *Inset:* kinetics of excitation of cGMP response, cAMP = 10^{-7}M (Redrawn from Van Haastert, 1987a). Panels B-E: Time course of cGMP formation in computer simulations according to different receptor models: Simple-adaptation, (panel B); Linear-adaptation, (panel C); Box-adaptation,(panel D); Circle-adaptation, (panel E). The concentrations of cAMP are: (1) 10^{-8}M, (2) 10^{-7}M, (3) 10^{-6}M.









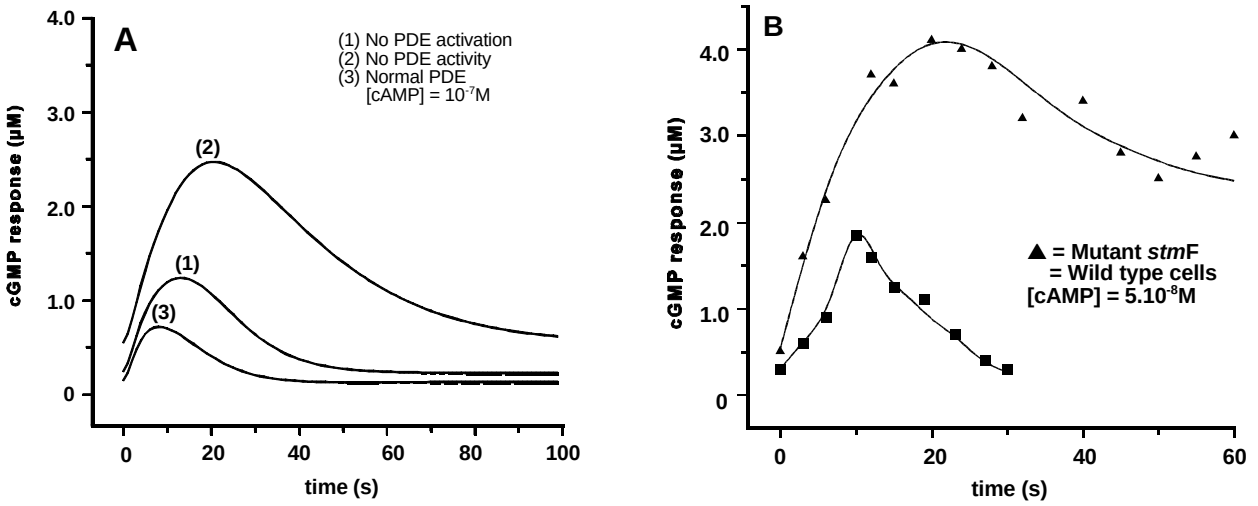


Figure 3. *Effect of cGMP-specific phosphodiesterase on the cGMP response.* Panel A, computer simulations performed with 10^{-7}M cAMP and different phosphodiesterase conditions. Panel B: experimentally observed cGMP response in *Dictyostelium* wild type and mutant *stmF*, which is defective in phosphodiesterase activity (redrawn from Newell, 1986)

This calculated response can be compared with experimental observations on strain HD10, which was obtained by disruption of the *Dictyostelium* phospholipase C gene; in this mutant cAMP induces the nearly normal cGMP accumulation (Drayer *et al.*, 1994). This suggests that the receptor-mediated activity of phospholipase C and subsequent expected release of Ca^{2+} does not significantly contribute to the cGMP response.

The removal of extracellular Ca^{2+} predicts a cGMP response that is 1.6-fold higher than the response of control cells (fig. 4A). Total depletion of Ca^{2+} inside and outside the cell gives a response that is 1.9-fold higher than the normal response. In both cases of changing the Ca^{2+} concentration, the kinetics of the response are unaltered; i.e. the peak is reached at the same time and cGMP levels recover with the same rate. When a constant intracellular Ca^{2+} concentration of 10^{-3} M is applied to the model, basal cGMP levels are reduced about four-fold and cAMP induces only a small cGMP response (about 35% of the normal response; fig. 4A). Experimental observations with electroporated cells in Ca^{2+} free buffer (HEPES/5.9 mM EGTA) show a large cGMP increase upon stimulation with cAMP (fig. 4B). Electroporated cells in the presence of 1 µM or 1 mM Ca^{2+} have reduced basal cGMP levels and show only a slight increase in cGMP levels after cAMP stimulation (fig. 4B).

DISCUSSION

Extracellular cAMP is a chemoattractant for *Dictyostelium* cells inducing cell aggregation and differentiation. Cells are stimulated by a wave of cAMP that is emitted from the aggregation centre. As the wave approaches the cell, the cAMP gradient has two characteristics: the cAMP concentration increases with time and the gradient points towards the

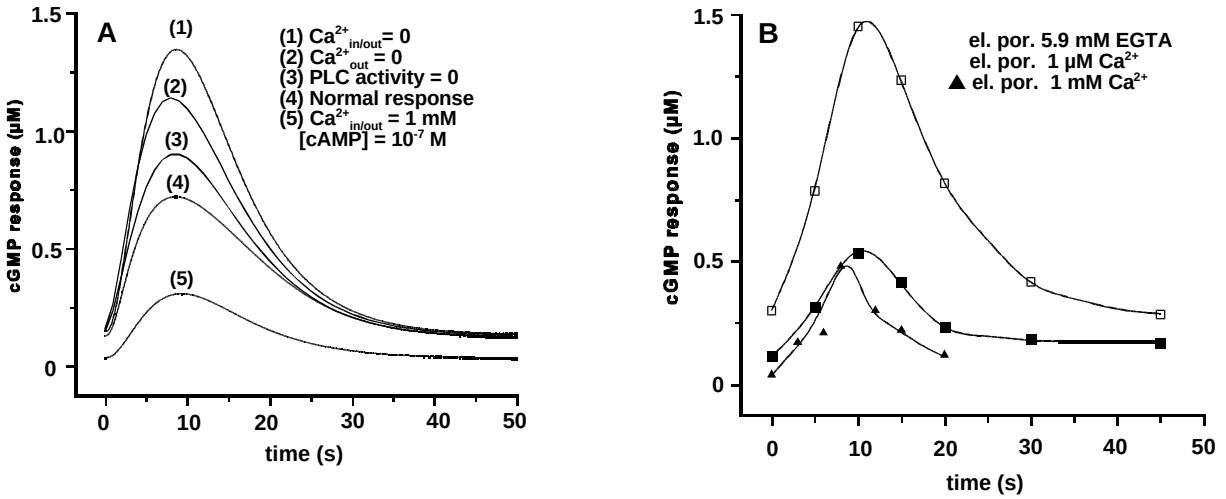


Figure 4. *Effect of Ca^{2+} ions on cGMP response.*
Panel A: Computer simulation of the cGMP response under varying Ca^{2+} concentrations or without phospholipase C activity. Panel B: experimentally observed cGMP response in electroporated *Dictyostelium* cells in HEPES/5,9 mM EGTA buffer (□), in HEPES/5,9 mM EGTA/5,9 mM $CaCl_2$ yielding 1 µM free Ca^{2+} (■), or HEPES/5,9 mM EGTA/6,9 mM $CaCl_2$ yielding 1 mM free Ca^{2+} (▲).

aggregation centre, leading the cell in this direction. When the maximal cAMP concentration of the wave passes the cell, both the spatial and temporal component of the cAMP gradient reverse: the direction of the gradient points away from the aggregation centre and the cAMP concentration decreases with time. If cells would respond to this concentration gradient, they would move away from the aggregation centre. Observations reveal that cells show directed movement on the rising flank of the cAMP wave and random movement after the wave has passed the cells (Alcantara & Monk, 1974). *Dictyostelium* cells extend pseudopods in the direction of the gradient within a few seconds upon stimulation with cAMP (Gerisch *et al.*, 1975). Rapid excitation in combination with perfect and rapid adaptation of the signal transduction cascade could explain the observations on directed cell movement when a cAMP wave passes the cells (Van Haastert, 1983c).

Chemotaxis is a complex reaction combining temporal and spatial information of the cAMP gradient. Several experiments suggest that the second messenger cGMP has an important function during chemotaxis. First, the kinetics of excitation and adaptation of the cGMP response are in good agreement with the kinetics of pseudopod formation during chemotaxis. Second, *stm*F mutants lacking a cGMP phosphodiesterase, show an enhanced cGMP response and prolonged chemotactic movement (Ross & Newell, 1981; Van Haastert *et al.*, 1982). Third, non-chemotactic mutants have recently been isolated, that do not respond to chemoattractants that are detected by different surface receptors; these KI mutants have a defect in the central sensory transduction cascade shared by different chemoattractants (Kuwayama *et al.*, 1993). Biochemical analysis reveal that most mutants show an altered cGMP response, varying from no guanylyl cyclase activity to an altered

balance between excitation and adaptation of cGMP formation.

The cAMP-mediated cGMP response in *Dictyostelium* is composed of a network of activation and adaptation of surface cAMP receptor, activation of guanylyl cyclase, inhibition of this enzyme by Ca^{2+} ions, and cGMP-stimulation of a cGMP-specific phosphodiesterases. We have studied the enzymes that are involved in the formation of the cGMP response. In order to understand the function of each of the components that participate in the cGMP response in relation to chemotaxis, a computer model for simulation experiments was designed, which is based almost entirely on experimental data. Detailed kinetic analysis of the components provides the framework for the model.

Four models on the adaptation of the receptor were investigated. Each model predicts different dynamics of the cGMP response, and only one model is in sufficient agreement with experimental data. This cyclic-adaptation model is based on observations on the interaction between cAMP and a subpopulation of receptors supposed to be coupled to the activation of guanylyl cyclase (Van Haastert *et al.*, 1986, Van Haastert, 1987c). The less favorable box-adaptation model that was proposed for the adaptation of adenylyl cyclase, predicts different kinetics of the cGMP response at different stimulus concentrations, which has not been observed experimentally. Two other more simple adaptation models, the Simple-adaptation model and the Linear-adaptation model, show insufficient adaptation. Although the cyclic adaptation model predicts all aspects of the cGMP response, its biochemical background is not completely understood. The interconversions of receptor forms that are observed *in vivo* can be induced by guanine nucleotides *in vitro*, suggesting that they are related to the altered interaction of the activated receptors with G-proteins.

The role of activation of cGMP-phosphodiesterase by cGMP was investigated in the model as well as experimentally. The model predicts that cGMP-phosphodiesterase affects both the magnitude and especially the duration of the cGMP response. Previous experiments with mutant *stmF*, which lacks the cGMP-phosphodiesterase, support the conclusions of the model. This suggests that cGMP phosphodiesterase functions by rapidly attenuating the cGMP response, even before guanylyl cyclase activity has completely recovered basal levels due to adaptation. During cell aggregation the cAMP concentration gradient directs movement to the aggregation centre for about 1.5 minute, which is the period that the cAMP concentration increases with time. In mutant *stm*F the cAMP gradient has probably the same concentration profile, but cells respond to the gradient for nearly three minutes. This suggests that cells continue to move in the same direction as long as cGMP levels are elevated, and that the function of the phosphodiesterase is to immediately erase the information contained in the cGMP response as soon as the cAMP concentration is no longer increasing with time.

Dictyostelium guanylyl cyclase is strongly regulated by nanomolar Ca^{2+} concentrations. Since the occupied surface cAMP receptor stimulates both guanylyl cyclase activity and an increase of cytosolic Ca^{2+} levels (Saran *et al.* 1994) (via Ca^{2+} uptake and possibly via IP_3-

mediated release from internal stores), the exact regulation of cGMP levels upon stimulation is not easily understood. Experiments on the effect of Ca^{2+} on cGMP levels in electropermeabilized cells reveal that Ca^{2+} reduces both basal and receptor stimulated cGMP levels, but has no strong effect on the duration of the response (Valkema & Van Haastert, 1992). The model predicts essentially this outcome, except that the effects of Ca^{2+} are stronger in the model than in the experiment. This notion is especially valid for the effect of removing extracellular Ca^{2+} and deletion of phospholipase C. In the model this will result in 1.7 and 1.3-fold increase of the response, but experiments reveal little effect of removing extracellular Ca^{2+} (Valkema & Van Haastert, 1992) or phospholipase C (Drayer *et al.*, 1994). This observation suggests that the regulation of guanylyl cyclase by Ca^{2+} may have been described appropriately, but that receptor-stimulated alterations of cytosolic Ca^{2+} concentrations are not completely understood. It should be noted that cytosolic Ca^{2+} levels have not been determined in detail upon cAMP stimulation of *Dictyostelium* cells.

In this study the dynamics of the cGMP response in time were investigated. Since chemotaxis combines temporal and spatial information of chemoattractant concentration, the next step will be to analyse the spatial distribution of cGMP during chemotactic movement. Unfortunately, cGMP levels cannot be measured yet in single cells, leaving only calculations to provide some insight in this process. The present investigations suggest that the main components that affect the kinetics of cGMP response have been identified. This information is now to be combined with estimated values for the spatial distribution of receptors, guanylyl cyclase and phosphodiesterase, and with diffusion of cGMP, IP_3 and Ca^{2+} inside the cell.

Summarizing, we conclude that receptor adaptation is responsible for the kinetics of the cGMP response. The activity of cGMP-stimulated cGMP-specific phosphodiesterase controls the magnitude and especially the duration of the cGMP response. The regulation of the guanylyl cyclase activity by Ca^{2+} ions gives *Dictyostelium* the opportunity for fine tuning of the cGMP response.

ACKNOWLEDGEMENTS

We thank Doekele Stavenga for introducing us to the computer simulation program Psi/e.

Table 1. Kinetic values of enzymes involved in the cAMP-induced cGMP response in *Dictyostelium discoideum*.

Constant	Unit	Value	Description	Ref.
η	-	0.8	fraction of GuCy that is sensitive for Ca^{2+}	[1]
n	-	2.3	Hill-coefficient of inhibition of GuCy by Ca^{2+}	[2,3]
K_I	M	$2x10^{-7}$	$[Ca^{2+}]$ giving half maximal inhibition of GuCy	[2,3]
δ	$M.s^{-1}$	$4.0x10^{-8}$	basal activity of GuCy	[4]
ε	$M.s^{-1}$	$1.7x10^{-6}$	activity of stimulated GuCy	[4]
V_G	$M.s^{-1}$	$2x10^{-6}$	hydrolytic activity of cGMP-specific PDE	[5]
V_A	$M.s^{-1}$	$2x10^{-7}$	hydrolytic activity of non-specific PDE	[6]
K_m^H	M	$5.4x10^{-6}$	K_m of activated cGMP-specific PDE	[5]
K_m^L	M	$2.4x10^{-5}$	K_m of basal cGMP-specific PDE	[5]
K_m^A	M	$2.5x10^{-6}$	K_m of non-specific PDE	[6]
k	$M^{-1}.s^{-1}$	$1.4x10^{-5}$	rate constant of activation of cGMP-specific PDE	[5]
k_-	s^{-1}	$2.0x10^{-2}$	rate constant of deactivation of cGMP-specific PDE	[5]
α	$M.s^{-1}$	$7.5x10^{-8}$	basal activity of PLC	[7]
ß	$M.s^{-1}$	$7.5x10^{-7}$	activity of stimulated PLC	[7]
	s^{-1}	$7.0x10^{-1}$	rate of degradation of IP_3	[8]
V_c^L	$M.s^{-1}$	$4.8x10^{-6}$	V_{max} of unstimulated Ca^{2+} channel	[9]
V_c^H	$M.s^{-1}$	$1.04x10^{-5}$	V_{max} of stimulated Ca^{2+} channel	[9]
K_m^{cL}	M	$1.15x10^{-4}$	K_m of unstimulated Ca^{2+} channel	[9]
K_m^{cH}	M	$1.85x10^{-5}$	K_m of stimulated Ca^{2+} channel	[9]
C	s^{-1}	$3x10^{-4}$	basal Ca^{2+} release from IP_3 sensitive store	[10]
D	s^{-1}	$3x10^{-3}$	as C, activated by IP_3	[10]
E	s^{-1}	6	rate of Ca^{2+} pump from cytosol to extracellular	[10]
F	s^{-1}	6	rate of Ca^{2+} pump from cytosol to store	[10]
M	-	2	Hill-coefficient of Ca^{2+} channel for IP_3	[10]
q	M	$7x10^{-7}$	K_m of Ca^{2+} channel for IP_3	[10]
$[Ca^{2+}]_{out}$	M	10^{-5}	$[Ca^{2+}]$ outside the cell	[11]
$[Ca^{2+}]_{store}$	M	10^{-3}	$[Ca^{2+}]$ inside the store	[12]
k_1	$M^{-1}.s^{-1}$	$2x10^{7}$	rate constant of association of cAMP to receptor R	[14]
k_{-1}	s^{-1}	$7x10^{-1}$	rate constant of dissociation of cAMP receptor complex	[14]
k_2	s^{-1}	$1.7x10^{-1}$	converting rate constant from R^*L to R^DL	[12]
k_{-2}	s^{-1}	$7.3X10^{-3}$	converting rate constant from R^DL to R^*L	[12]
k_3	s^{-1}	$5.78x10^{-4}$	converting rate constant of R^S to R^D	[13]
k_{-3}	s^{-1}	$5.2x10^{-3}$	converting rate constant of R^D to R^S	[13]
k_4	s^{-1}	$1.6x10^{-1}$	converting rate constant of R^DL to R^SL	[13]
k_{-4}	s^{-1}	$1.73x10^{-2}$	converting rate constant of R^SL to R^DL	[13]
a_1	-	$1.0x10^{-1}$	specific activity of R^S	[13]
a_2	-	1	specific activity of R^SL	[13]
a_3	-	$1.9x10^{-4}$	specific activity of R^DL	[13]
a_4	-	$7.0x10^{-2}$	specific activity of R^D	[13]
K_R	M	$1.5x10^{-8}$	dissociation constant of R^SL	[13]
K_D	M	$1.5x10^{-8}$	dissociation constant of R^DL	[13]
k_x	s^{-1}	$0.22e^{-0.17t}$	converting rate constant from RL to R^*L	[14]
k_y	s^{-1}	$1.7x10^{-1}$	converting rate constant from R^*L to R^DL	[14]
k_z	s^{-1}	$7.3x10^{-3}$	converting rate constant from R^DL to RL	[14]

References [1] Van Haastert, unpublished observations; [2] Janssens *et al.* 1989; [3] Valkema and Van Haastert 1992; [4] Mato and Malchow 1978; [5] Van Haastert and Van Lookeren Campagne 1984; [6] Malchow *et al.* 1972; [7] Bominaar *et al.* 1994; [8] Van Lookeren Campagne *et al.* 1988; [9] Millne and Coukell 1991; [10] Champeil *et al.* 1989, Streb *et al.* 1983; [11] Bumann *et al.* 1984; [12] estimated; [13] Based on Knox *et al.* 1984; [14] Van Haastert *et al.* 1986

The role of guanylyl cyclase

In search of the *Dictyostelium discoideum* guanylyl cyclase gene

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Chapter 4 - part III

ABSTRACT

In *Dictyostelium* the enzyme guanylyl cyclase, the second messenger cGMP synthesizing enzyme, is one of the key enzymes in signal transduction. We here describe the strategies employed in the search for the gene that codes for guanylyl cyclase. The cDNA of the *Strongylocentrotus* guanylyl cyclase gene was used as a probe in Southern blot experiments, and a degenerated primer based on conserved regions of known sequences was used to screen a λGT11 library. Also, degenerated primers were used in polymerase chain reactions. Furthermore, we tried to identify the gene by an *E. coli* complementation system, in which cGMP is necessary for the organism to grow on maltose. Finally, we screened an expression library with a polyclonal antibody directed against a region of the conserved catalytic domain of the bovine lung soluble guanylyl cyclase.

Although many new genes were cloned, none of them appeared to encode a putative guanylyl cyclase. During the preparation of this thesis, members of the research group cloned a gene that shows all the characteristics of a guanylyl cyclase. Inspection of the amino acid and DNA sequence explains why the strategies described in this chapter were unsuccessful. The recent cloning of the putative guanylyl cyclase is described in the appendix.

INTRODUCTION

Since the identification of cyclic-GMP (cGMP) in rat urine in 1963 (Ashman *et al.,* 1963) an important role in signalling responses has been attributed to this second messenger molecule. In vertebrates, these responses include smooth muscle relaxation, natriuresis, phototransduction, inhibition of hormone secretion and stimulation of intestinal fluid secretion (reviewed in Drewett & Garbers, 1994). In invertebrates, protection against osmotic stress, enhanced sperm motility and respiration induced by egg peptides are all associated with increases in intracellular cGMP levels (Shimomura *et al.*, 1986; Kuwayama *et al.,* 1996). In each case, elevation of cGMP levels is due to the activation of soluble or membrane forms of guanylyl cyclase by extracellular stimuli. The increased cGMP amounts result in activity changes of target molecules, such as phosphodiesterases, cGMP dependant protein kinases, and ion channels, which finally generate the physiological responses of the cell (Lincoln and Cornwell, 1993).

Guanylyl cyclases are found both in the particulate fraction and the soluble fraction of the cell. Based upon their topography the guanylyl cyclase subtypes are classified into two general structural models. Membrane associated guanylyl cyclases share a common motif featuring a putative transmembrane domain, flanked by a kinase-like and cyclase catalytic domain that point into the cellular interior and an extracellular peptide binding domain. Active soluble guanylyl cyclases exist of heterodimers, of which the α and β subunits contain the conserved cyclase catalytic domain. The cyclase catalytic domain in all guanylyl cyclases shows sequence similarity to the catalytic domains in adenylyl cyclase.

Different types of ligand have been identified as direct activators of guanylyl cyclase activity. Nitric oxide (NO) was found to activate soluble forms of guanylyl cyclase. Some soluble guanylyl cyclases associate with a heme group, which is

necessary for regulation, probably by serving as a recognition site for NO (Craven & DeRubertis, 1978, Gerzer *et al.*, 1981). Transmembrane guanylyl cyclases have a peptide binding domain in their extracellular region that is involved in mediating cyclase activity (Duda *et al.*, 1991). Ligands that stimulate cGMP formation are: from sea urchin the sperm stimulating peptides, speract and resact (Garbers, 1989); from mammalian tissues the atrial natriuretic peptides (ANP), classified in three groups as types A,B and C (Hamet *et al.*, 1984, Waldman *et al.*, 1984, Winquist *et al.*, 1984); the heat-stable enterotoxin STa, product from some strains of pathogenic bacteria (Schultz *et al.*,1990); and the oligopeptide guanylin, isolated from adult rat jejunum (Weigand *et al.*, 1992).

In *Dictyostelium discoideum* the presence of a guanylyl cyclase is predicted from observed receptor mediated elevations of the basal cGMP level in sensitive cells. When folic acid is offered to post-vegetative cells, or cAMP to aggregative cells, a transient increase of cGMP production is detectable. This cGMP wave reaches its maximum after ten seconds and returns to basal concentrations after thirty seconds. Much is known about the regulation of this guanylyl cyclase activity and the kinetics of the cAMP mediated cGMP response, however, the identity of the gene coding for *Dictyostelium* guanylyl cyclase remained unclear. We have applied a variety of molecular approaches to obtain (part) of the DNA coding sequence. In this report we present the review the different strategies.

MATERIALS AND METHODS

Strains

Dictyostelium discoideum wild-type AX3 were grown at 22 °C in HG5 medium. Aggregation competent cells were obtained by starvation of cells in late logarithmic phase for 5 hours on a rotary shaker in 10 mM KH_2PO_4/Na_2HPO_4 pH 6.5 (PB) at a cell density of 10^7 per ml. Guanylyl cyclase mutants KI8 and KI10 were grown on 1/3 SM plates (0.3% glucose, 0.3% bactopeptone, 1.5% agar and 40 mM KH_2PO_4/Na_2HPO_4 pH 6.0) with *Klebsiella aerogenes*. *E.coli* cells TP2073 cells, a kind gift from Dr. Beuve (Paris), were grown on rich LB medium or on M63 medium (Miller *et al.*, 1972).

Materials

A λgt11 cDNA library, derived from mRNA from aggregation competent cells (Klein *et al.*, 1988) was a kind gift from Dr. Devreotes, Baltimore. A genomic λZAP library was a gift of Dr. Ennis, New York. Genomic DNA from *Dictyostelium* strain AX3 and NC4 was isolated as described by Nellen *et al.* (1988). The pBR322 library was made by ligation of *Sau*IIIA partially digested chromosomal *D. discoideum* DNA into the *Bam*HI site of plasmid pBR322.

Restriction enzymes and DNA ligase were from Boehringer, Mannheim. *Taq* polymerase was from Promega.

Southern blotting with 600-bp sea-urchin GC probe

A 600-bp *Stu*I-*Pst*I fragment from sea-urchin *Strongylocentrotus purpuratus*

Figure 1. *Alignment of known guanylyl cyclases and adenylyl cyclases*

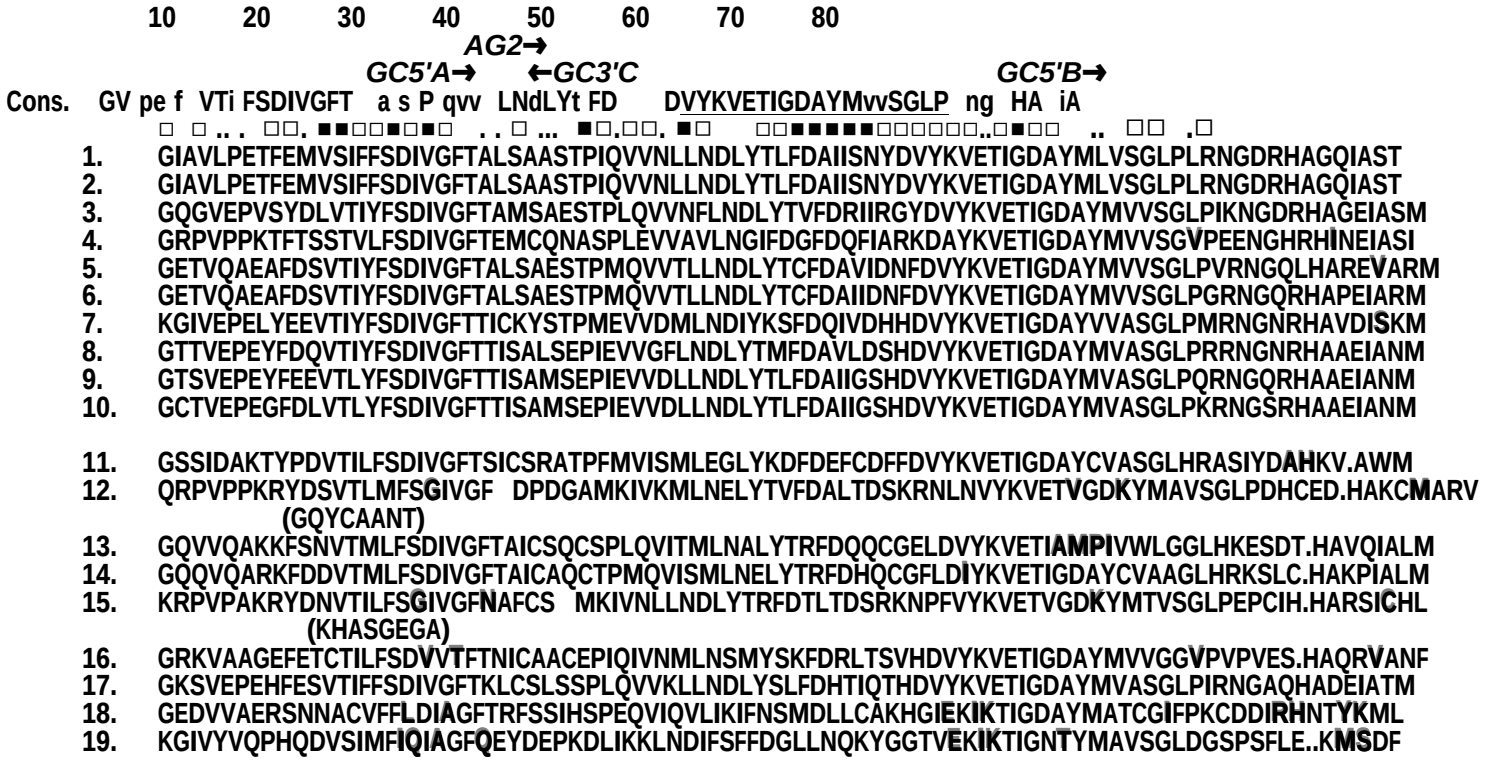

Figure 1. *Comparison of part of the amino acid sequence of 17 guanylyl cyclases and 2 adenylyl cyclases.*
In uppercase residues that are conserved in all GC'S (■) or in at least 14 out of 17 GC's (□); in lowercase conserved residues in more than 10 GC sequences (•). In bold, residues that are a deviation from consensus sequence, underlined is the region after which the peptide GC5 was designed, used to raise a polyclonal antibody. The starting positions of PCR primers are marked with arrows (→←) Numbers refer to the following proteins **Membrane bound GC's (1-10)** 1. *Hemicentrotus Pulcherrimus* GC; 2. *Strongylocentrotus Purpuratus* membrane GC; 3. *Drosophila* GC receptor; 4. *C. Elegans* GC similar to receptor type GC; 5. Rat Atrial Natriuretic Peptide receptor; 6. Bovine Atrial Natriuretic Peptide receptor B precursor; 7. Rat GC toxine receptor; 8. Rat receptor GC; 9. Rat GC E precursor; 10. Rat GC F precursor; **Soluble GC's (11-17)** 11. *Drosophila* soluble GC, alpha subunit; 12. *Drosophila* soluble GC, beta subunit; 13. Human soluble GC; 14. Human soluble GC alpha-2 subunit; 15. Human brain soluble GC; 16. Rat soluble GC from kidney; 17. Rat soluble GC; **Adenylyl cyclases (18-19)** 18. Dictyostelium adenylyl cyclase germination stage 19. Dictyostelium adenylyl cyclase aggregation stage, second domain.

Figure 1. (continued)

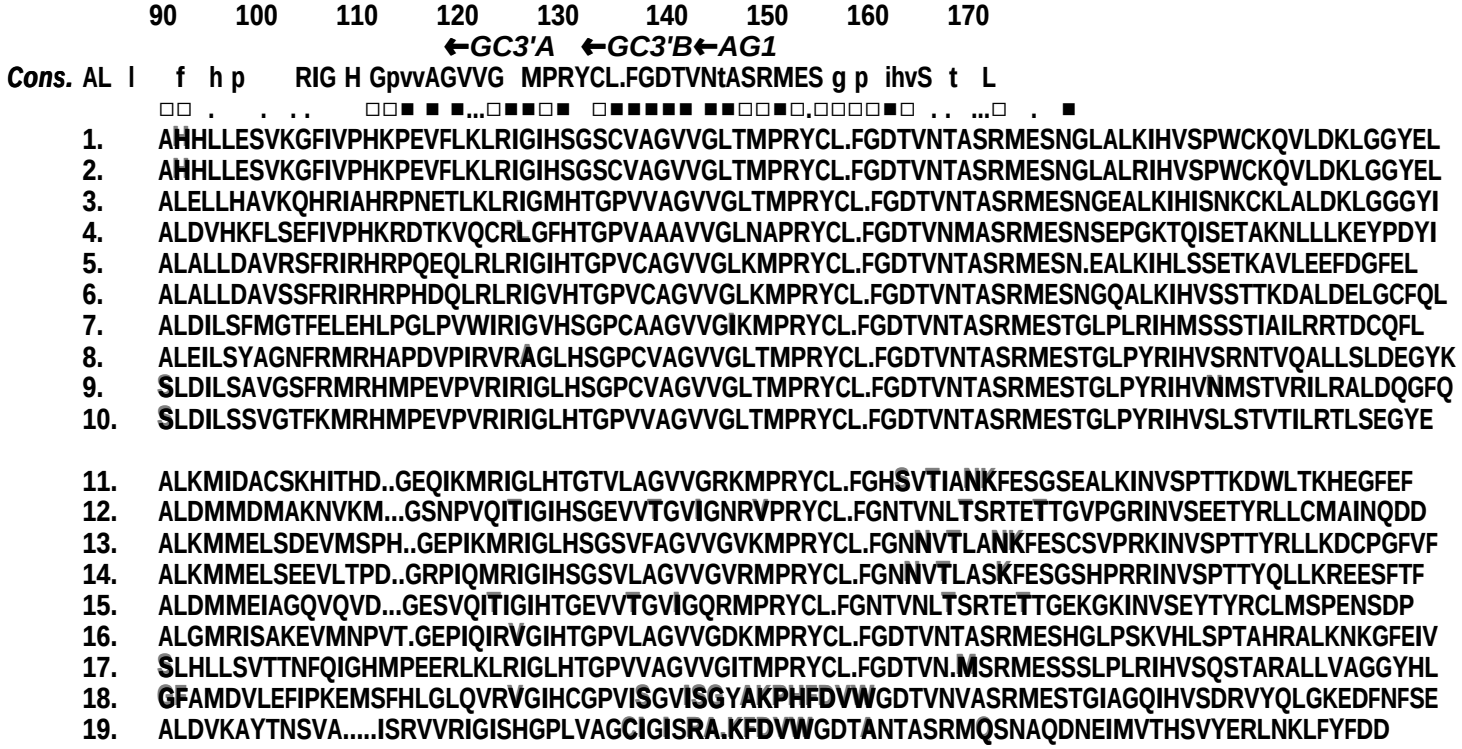

Figure 1. *Comparison of part of the amino acid sequence of 17 guanylyl cyclases and 2 adenylyl cyclases (continued)*.
References: [1] Shimizu *et al.*, 1993, [2] Thorpe *et al.*, 1989; [3] McNeil, *et al.*, 1995; [4] Wilson *et al.*, 1994; [5] Yamaguchi, 1990; [6] Fenrick *et al.*, 1994; [7] Schultz *et al.*, 1990; [8] Fulle *et al.*, 1995; [9], [10] Yang *et al.*, 1995; [11], [12] Shah *et al.*, 1995; [13],[15] Giuili, *et al.*, 1992; [14] Behrends *et al.*, 1995; [16] Yuen *et al.*, 1990; [17] Kojima *et al.*, 1995. [18, 19] Pitt *et al.*, 1992 .

(Thorpe *et al.*, 1989) was isolated and randomly labelled with the High-prime labelling kit (Boehringer, Mannheim). Southern blots of *HindIII*, *EcoRI* and *HindIII/EcoRI* digested *Dictyostelium* AX3 genomic DNA on nitrocellulose were hybridized 17 h with radiolabeled probe at 42 °C in 4.8 x SSC, 1 x Denhardt's solution, 10% dextransulphate, 0.1% SDS, 0.02M Tris.Cl, pH 7.6, 60 µg/ml denatured herring sperm DNA supplemented with 0%, 15%, 30% or 50% formamide. Nitrocellulose filters were rinsed at room temperature in 6 x SSC, 0.5% SDS and washed at 42 °C.

λGT11 cDNA screen with oligonucleotide

The antisense degenerate oligonucleotide GC3'C, complementary to a conserved region of known guanylyl cyclases was designed as follows: GC(A/G)(G/T)T(A/G)AAACC(A/G)AC(A/G)ATA(C/T)C.

Approximately $5x10^4$ recombinant phages from a *Dictyostelium* λgt11 cDNA library on nitrocellulose filters were hybridized for 17 h with ^{32}P labelled oligonucleotide probe as described above. Autoradiography was for 18 h at -80°C, with Kodak XAR-5 film and intensifying screens. Phage DNA was isolated by elution over Qiagen columns (Qiagen Inc.). Inserts were excised by *KpnI/SacI* double digestions and ligated into pBlueScript S/K vector for sequencing.

DNA amplification using the polymerase chain reaction

Degenerated oligonucleotides complementary to conserved regions of known guanylyl cyclase genes (fig. 1) were designed taking into account the codon usage in *Dictyostelium.* The oligonucleotide sequence of the sense primers used, were as follows:

GC5'A: (5'-GGTCTAGA(T/C)AT(A/T/C)GT(A/T/C)GG(A/T/C)TT(T/C)A(A/C)(A/T/C)GC),

GC5'B: (5'-CCGAATTCGT(A/T/C)TA(T/C)AA(G/A)GT(A/T/C)GA(G/A)AC)

and AG2:(5'-AACGAATTCAA(G/A)(G/A)T(A/T/G)(A/G)A(A/G)AC(A/T/C)AT(A/T/C)GG).

As antisense primers the following oligonucleotides were used:

GC3'A: (5 ' - AAGGATCCA(A/G)(A/G)CA(A/G) TA(A/T)C(T/G)(A/T)GGCAT),

GC3'B: (5'-GGAAGCTTA(A/G)(A/T/G)CC(A/T/G)AC(A/T/G)AC(A/T/G)CC(A/T/G)GC) and AG1: (5 ' - TACCCATGGC(A/T/G)GT (A/G)TT(A/T/G)AC(A/T/G)(T/G)T(A/G)T(T/C) (A/T/G)CC).

The 5'-end of each primer was provided with a restriction site and at least two extra nucleotides. PCR reactions were performed with *Taq* polymerase from Promega.

Expression library screen

The polyclonal antibody GC5 and the corresponding antigenic oligopeptide $GC_{pep}5$, directed against the amino acid sequence VYKVETVGDKYMTVSGLP were a kind gift of Dr. Koesling, Berlin.

Cells from host strain *E. coli* Y1090 were infected with λGT11 cDNA, incubated at 42 °C for 3.5 h and covered with nitrocellulose filters (Schleicher & Schuell) that were immersed in 10 mM IPTG, and incubated for an additional 3.5 h at 37 °C. Membranes were washed in TBST (50 mM Tris.Cl, pH 8.1, 150 mM NaCl and 0.05% Tween-20) and blocked in TBST with 5% low fat dry milk. Membranes were incubated 90 min. with a 1:600 dilution of the GC5 antibody (optionally preincubated with $GC_{pep}5$) in

TBSTG (TBST with 0.1% gelatine). As secondary antibodies were used horseradish peroxidase labelled anti-rabbit-IgG antibodies, in a 1:50,0000 dilution in TBSTG or alkaline phosphatase labelled anti-rabbit IgG antibodies in a 1:2000 dilution in TBSTG. The blots were detected with the ECL Western blotting system from Amersham (UK) or by alkaline phosphatase activity assay according to Sambrook *et al.* (1989). The filters were washed three times with TBST for 5 min between different incubation steps.

SDS - PAGE and Western blotting

Dictyostelium cell lysates were denatured in SDS-gel loading buffer at 100°C for 5 min and separated by gelelectrophoresis according to Laemmli (1970). The proteins were transferred to nitrocellulose filters by electroblotting in Tris.Cl buffer (25 mM, pH 8.3) containing 192 mM glycine, 0.02% SDS and 20% methanol at 200 mA for 1.5 h. Filters were further treated as in the expression library screen. Antigenic bands were visualized with alkaline phosphatase coupled second antibody.

RESULTS AND DISCUSSION

cDNA library screen

All guanylyl cyclases share sequence similarity of their catalytic domains (fig. 1). The first experiments were aimed at screening a *D. discoideum* cDNA library using a radiolabeled DNA probe coding for the catalytic domain of guanylyl cyclase, derived from a previously cloned gene. Genomic DNA of *Dictyostelium discoideum* has an extremely low GC/AT ratio (30/70) compared with DNA of many other organisms. Before screening the library we first performed Southern blot analysis using a GC DNA probe from *Strongylocentrotus purpuratus,* a lower eukaryote like *Dictyostelium*, with comparable GC/AT ratios. The filters were probed with a 600-bp *StuI/PstI* fragment of *Strongylocentrotus* cDNA coding for the guanylyl cyclase catalytic domain. Using buffers of different stringencies at 42 °C the radiolabeled probe did not show specific hybridization with the *Dictyostelium* genomic DNA. From this we concluded that the nucleotide similarity between the *Dictyostelium* guanylyl cyclase gene and that from *Stongylocentrotus* is too small to allow screening a cDNA library. Although *Strongylocentrotus* is a lower eukaryote like *Dictyostelium*, it probably evolved just 20 million years ago. *Dictyostelium* is believed to have branched off a billion years earlier (Loomis & Smith, 1995). Probably the two organisms have diverged that much that the similarity between the *Strongylocentrotus* guanylyl cyclase gene and the *Dictyostelium* counterpart has reduced enough to prevent hybridization of related genes.

Another approach to screen the cDNA library was based on using an oligonucleotide probe derived from a small hyperconserved area from the consensus of the guanylyl cyclase catalytic domain (fig. 1). Since hybridization of a 20-mer oligonucleotide with cDNA on a nitrocellulose filter will be overshadowed by a reasonable background we aimed to design a primer that would be minimally degenerated. The antisense primer GC3'C corresponding to the amino acid region (G/D)IVGF(T/N)A was employed in Southern blot analysis to establish the ideal buffer stringency and hybridizing conditions. We detected a specific band with an acceptable

```
EFVYKVETVTGKPLVAYRETIQQ-RGDYNFTTRKQSGGQGQYATILFGS          clone 71
  ::  ::: :::::::: :   :     ::::: ::
KREFNVEANVGKPQVAYRETIRQKVTDVEGKHAKQSGGRGQYGHVVIDMYPLEP    EFT Ecoli
```

Figure 2. Amino acid translation of clone 71 The open reading frame is compared with the amino acid sequence of elongation factor theta from E. coli. The sequences show 42% identity in a 48-bp overlap.

background when hybridisation was performed at 42°C in a 0% formamide buffer. Under the obtained conditions we screened 50,000 plaques, which yielded 22 positive clones. After purification of the λGT11-clones the cDNA insert was obtained by PCR amplification using the λ-forward and λ-reverse primer. After subcloning into the *EcoRI* site of a pBluescript vector five different λ-inserts were sequenced. Careful analysis of the inserts revealed no clones having homology with known guanylyl cyclases. In four cases the products that were found showed homology with 40S and 60S ribosomal proteins. Furthermore one fragment showed homology with a glutathione S-transferase from maize (data not shown). In the amino acid sequence of the isolated fragments, the (G/D)IVGF(T/N)A sequence could not be detected. However, in the DNA sequence of the non-coding complementary DNA strand we found a match of 75% in a stretch of 16 base-pairs. Given the low hybridizing temperature and a low stringency buffer, this may explain why the particular clones were picked up.

PCR

For a second set of experiments we analysed the amino acid sequences of the catalytic domains of cloned guanylyl cyclases and adenylyl cyclases to design primer sets that were used in polymerase chain reactions. The region considered being the catalytic domain is the most conserved part in both guanylyl and adenylyl cyclases. Figure 1 shows an alignment of the partial structure of 17 different guanylyl cyclase genes and two adenylyl cyclases that were cloned thus far. In this sequence alignment it appears that several stretches are highly conserved. The sense primers GC5'A and GC5'B correspond to amino acid regions DIVGFT and VYKVET, the antisense primers GC3'A and GC3'B correspond to amino acid regions AGVVGL and MPRYCL.

With PCR several DNA fragments of the desired length were isolated and subcloned in a sequencing vector. The cloned fragments were sequenced and the amino acid translations of the DNA sequences were compared to previously published genes in the EMBL databank. PCR on cDNA with the primer combination VYKVET/MPRYCL thus gave a product of the expected length of 248-bp, of which the nucleotide sequence was determined. Among the sequenced clones several shorter and longer open reading frames were detected, which were compared with the EMBL protein bank. Most of the fragments did not have any resemblance to known genes. Worth to mention is one clone of which the fragment showed 42% identity in 48 aa overlap with an Elongation factor F homologous protein (fig. 2). The approx. 248-bp product was also used as a template for PCR with the primer combination VYKVET/AGVVGL, which

yielded the expected 236-bp product. The non-purified product was radio-

```
GLYTGFGVSVGGIFVYRAAFFGGYDTAKGILLGENNKKASFWASWGIAQVVTTIAGVVRLPKRY  clone 105
::: :: ::: :: ::::: :: :::::: :     :     :: ::: :   ::::
GLYQGFSVSVQGIIIYRAAYFGVYDTAKGML--PDPKNTHIVVSWMIAQTVYAVAGVVSYPFDT  adt3 human
```

Figure 3. Clone 105: comparison of open reading frame with amino acid sequence of ADT3, an ADP-ATP carrier protein from human liver. Sequences show 59% identity in a stretch of 61 amino acids.

labeled and used to screen a cDNA-library. This revealed 16 clones with inserts varying from 500 to 1000-bp. After sequencing, fifteen clones were found to be identical, showing homology with human mitochondrial ADP-ATP carrier protein from liver (fig 3). In the C-terminal part of this product we see the amino acid sequence AGVVSY, which explains why the primer AGVVGL could anneal with this DNA.

The PCR primers AG1 (K(I/V)(K/E)TIG) and AG2 (G(N/D)(T/N)VNTA) were designed after the mutual characteristics of the conserved regions in the catalytic domains of guanylyl and adenylyl cyclases. Amplifications of chromosomal DNA with this primer set gave a 250-bp product that was subcloned and sequenced. Four of the obtained clones contained part of the DNA that codes for one of the two catalytic domains of the adenylyl cyclase A, isolated previously, proving that this strategy was successful. Translation of the DNA insert of another clone showed an open reading frame of 87 amino acids (fig. 4). Though some amino acids of the PCR product agreed with conserved amino acids in guanylyl cyclases, isolation of the flanking cDNA regions demonstrated that none of the other conserved regions could be found in this gene. Many of the sequenced inserts of this PCR reaction, however, were primer concatamers that frustrated the investigation of a larger number of gene fragments.

Immunological screen of an expression library

The third set of experiments were performed by aiming to clone the GC gene by screening an expression library

```
          10        20        30        40        50
            □□•  •□     □
KIETIGLLDGLPVRQTGRLASDPGFDLGFMTLAEGQTVATAEKYLNNHLE

          60        70        80

ITIFYHSNPTDNTSRIVGFEIFPTSRQYKKVENWKGDTV
```

Figure 4. Amino acid sequene of clone AG14, primer sequences are marked in bold. Some amino acids are homologous to the guanylyl cyclase sequence, ( ): conserved in all GC's, (□): conserved in at least 14 out of 17 GC's; (•): conserved in 9 known GC's.

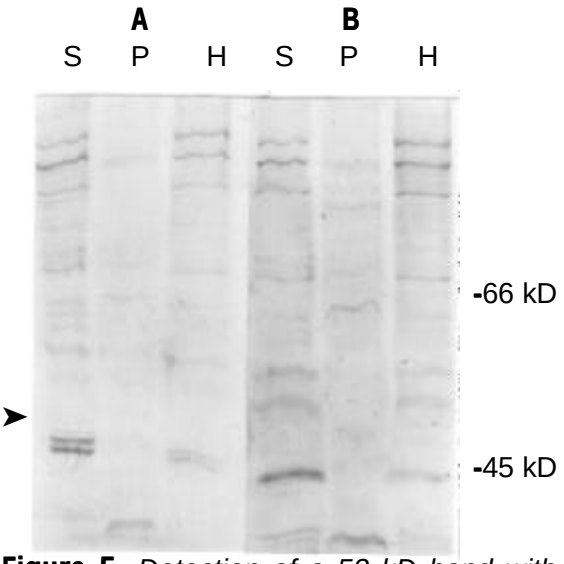

Figure 5. *Detection of a 53 kD band with Western analysis*
AX3 cells were grown at 22°C until mid log phase, washed and starved for 5 hours in PB. Whole cell lysates were analysed by Western blot using GC5 antibodies and visualized by the alkaline phosphatase labeled second antibody. The GC5 antibodies were incubated without (A) and with (B) peptide. S: soluble fraction; P: pellet fraction; H: homogenate. Arrow: 53 kD band.

with a polyclonal antibody directed against an 18 amino acids long conserved region of known guanylyl cyclases (Guthman *et al.*, 1992). Usually, polyclonal antibodies show nonspecific interaction with complex protein solutions like cell lysates. Before screening the library, Western blots of *Dictyostelium* cell lysates were incubated with the GC5 antiserum to investigate the specificity of the antiserum. As a control, the GC5 antiserum was preincubated with the peptide (20 µg/ml) that was used as antigen. To identify a potential guanylyl cyclase band, one filter was incubated with antibody to show all antigenic bands. A duplicate filter was incubated with antibody under addition of the oligopeptide $GC5_{pep}$ to prevent the specific antibodies to couple to their antigenic proteins, thus marking the missing band as the guanylyl cyclase band.

Experiments with cell lysates of wild type AX3 cells showed a band of 53 kD, present on filters incubated with antibody, but clearly absent on filter duplicates incubated with antibody GC5 preincubated with 20 µg oligopeptide (fig. 5). Separation of the soluble fraction from the particulate cell fraction showed that the antigenic protein resided in the cytosolic fraction. Previous experiments of Schulkes *et al.* (1993) indicated that guanylyl cyclase activity mainly is found in the particulate fraction of the cell. However, activity in cell lysates is lost within 30 min, which can be due to a break in the connection between guanylyl cyclase protein and a factor that is necessary for the activation of its activity (Schulkes *et al.*, 1993). We cannot rule out the possibility that guanylyl cyclase is only active when it is associated with the membrane, and that a minor part is really associated with the membrane, leaving the bulk in the cytosolic fraction.

Next we tested the efficiency of the antibodies with *Dictyostelium* guanylyl cyclase mutants KI8 and KI10. KI8 is a guanylyl cyclase minus mutant, whereas KI10 synthesizes a guanylyl cyclase with an activity that cannot be stimulated by receptor induction. Incubation of antibody GC5 with cell lysates of these mutants however, showed an antigenic pattern, which did not differ from the pattern of AX3 lysates. Though both mutants have an aberrant guanylyl cyclase activity, it has not been proved the protein is not synthesized in KI8 and KI10. The result indicated however that unambiguously identifying antigenic spots in an expression library would be difficult.

In seven separate experiments 480,000 pfu were screened which yielded 10

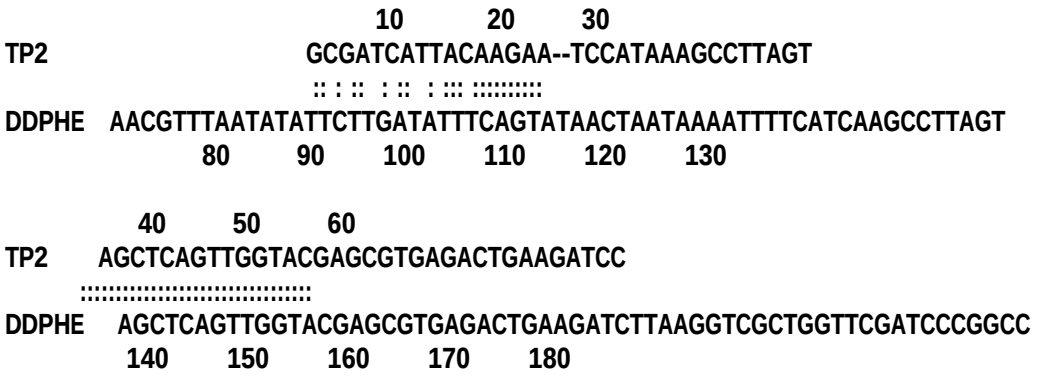

Figure 6. Comparison of the nucleotide sequence of clone TP2 with the *Dictyostelium discoideum* gene for phenylalanine tRNA (DDPHE) The obtained TP2 fragment shows 85% identity over a stretch of 65 nucleotides to the sequence of the DDPHE gene.

clones that reacted with the GC5 antibody. Incubation of the duplicate filter with the GC5 antibody pre-incubated with the $GC5_{pep}$ revealed that the 10 clones were false positives. At this stage we decided that identifying a clone expressing part of guanylyl cyclase protein by Western analysis using the GC5 antibody would not succeed.

Guanylyl cyclase complementation system

Guanylyl cyclase and adenylyl cyclases synthesize different but related products and it is commonly believed that the enzymes have evolved from a common ancestor (Krupinski *et al.*, 1989). In *E.coli*, maltose fermentation is induced by the cAMP-activated receptor, encoded by the *crp* gene. Beuve *et al.* (1992) obtained an *E.coli* strain TP2073, which has two important mutations: it is deficient in adenylyl cyclase, and the mutated *crp* gene product recognizes cGMP instead of cAMP. Thus, the mutant strain, depends on cGMP formation for maltose fermentation (table 1), which allows this strain to be used for selecting guanylyl cyclase upon transformation with an expression library.

Attempts to infect *E. coli* TP2073 cells with λZAP failed, possibly due to rapidly disappearing maltose receptors, which are necessary for infection by λ phage particles. We therefore transformed *E.coli* TP2073 cells with a *Dictyostelium* pBR322 genomic DNA library, to select for mutants that can grow on minimal medium with maltose, to find the DNA coding for guanylyl cyclase activity. TP2073 cells transformed with empty pBR322 plasmid showed growth on LB media with ampicillin and low concentrations of cGMP (10 µM) or cAMP (less tan 10 µM) (table 2). Higher concentrations of cAMP are toxic to the cells, but not in the presence of cGMP. The transformants

Table 1. *E. coli TP2073 growth on several media*

	Growth in presence of		
Media	–	cGMP	cAMP
LB	+	+	–
LB + ampicillin	–	–	–
M63 + glucose	+	+	–
M63 + maltose	–	+	–
M63 + arabinose	–	+	–

Table 2. *Growth of E. coli TP2073 cells transformed with plasmids (pBR322 +/– Dd genomic DNA) on several media*

Media	Growth after transformation with plasmid	
	pBR322	pBR322 + insert 1-8
LB + Amp + 0.25mM cA	–	+
LB + Amp + 0.25 mM cA + 10 µM cG	+	+
M63 + Amp + glu	+	+
M63 + Amp + mal	–	+

showed growth on minimal medium with glucose, however when maltose was added to the medium pBR322 transformants did not survive. In this way we acquired a selection system that could identify cGMP producing clones.

The plasmid DNA from clones that survived on minimal medium with maltose, carrying pBR322 with insert, was isolated and sequenced. Eight clones were isolated this way, carrying inserts varying from 300-2000-bp. Comparison of the sequences of the obtained inserts with other DNA sequences revealed that cloned products contained contaminations from vector sequences from pGEM7 and pBR322. Interestingly, clone 2 with an insert of 300-bp showed an identity of 85% over 65-bp with tRNA-Phe from *Dictyostelium discoideum* (fig 6). This tRNA, which is encoded by a 300-bp gene, is associated with 25-bp of 3'-terminal C-modul of the retrotransposon DRE (Marschalek *et al.*, 1992).

Yet, the reason why clone 2 could rescue the phenotype of TP2073 remains unclear. Survival of the mutant TP2073 on minimal media with maltose could be due to a spontaneous mutation. We did not transform TP2073 cells with the obtained DNA, to rule out this possibility and to find out whether the plasmid DNA of this clone could rescue the mutant phenotype. The complementation system appeared to be insufficiently selective and sensitive for obtaining the guanylyl cyclase gene from *D. discoideum*. We here decided that trying to find the gene with a complementation strategy like the TP2073 system would not be successful and the experiments were stopped at this stage.

The attempts in finding the gene for guanylyl cyclase of *Dictyostelium* presented above, all were in vain. However, finding the gene is only a matter of time. The most promising strategy to isolate the GC gene will be PCR. Almost 30 new GC genes were cloned during the search for the particular *Dictyostelium* gene, and the knowledge of the conserved regions in the catalytic domain therefore increased as well. The waiting is for new genes that have little irregularities compared with the classical GC genes, thus showing the final strategy for PCR primer design.

APPENDIX

In recent experiments, which will be published in detail later, our lab succeeded in identifying part of the gene coding for the putative *Dictyostelium discoideum* guanylyl cyclase by means of PCR. The primers were based on conserved regions of known guanylyl cyclases, separated by only three amino acids. After sequencing, the PCR product was used to screen a λGT11 library from which a 1050-bp insert was obtained that translated to the C-terminal part of a protein. The deduced amino acid sequence shows significant homology to other guanylyl cyclases (shown in fig. 7). Compared with the GC consensus of the catalytic domain we calculated an identity of 33% (table 3). When we compare the obtained sequence with the consensus of known GC's, the sequence shows number of the hallmarks which makes it a guanylyl cyclase more than an adenylyl cyclase. The cloned fragment hybridizes in a Northern blot with a 4-kb mRNA.

In figure 7 the underlined amino acids indicate the stretches after which the primers were designed, which were used before for PCR and λ library screen. Comparing those primers with the obtained DNA sequence, the most striking differences are the two primers GC5'B (VYKVET) and GC3'A (MPRYCL) which both have three deviant amino acids, resulting in 5 and 8 nucleotides mismatch respectively, that prevented the two designed primers from proper annealing.

Also the primer GC5'A (DIVGF(T/N)A) has an important nucleotide mismatch at the 3' position, where we designed an alanine (A) and the *Dictyostelium* guanylyl cyclase gene codes for a glutaminate (E). Primer GC3'B (AGVVGL) also has a nucleotide mismatch at the 3' position, where we designed for a leucine (L), but which appeared to be an isoleucine (I). This mismatch at an antisense primer would not have to be fatal. However, since none of the other sense primers used in PCR could anneal with matching sequences, no product could be found using any combination of the designed PCR-primers. The GC3'C primer used in the λGT11 library screen showed 6 mismatches on DNA level with the cloned product, too many to hybridize with each other under the experimental conditions.

Of the AG primers, which are based on amino acid consensus sequences of both adenylyl cyclases and guanylyl cyclases, AG2 makes the best match, with only one

Table 3. *Identity of the putative Dictyostelium guanylyl cyclase gene fragment compared with the consensus of catalytic domains of guanylyl cyclases and adenylyl cyclases, and with the consensus of Dictyostelium adenylyl cyclases ACA, domain 2 and ACG.*

	% identity			
	GC consensus	AC consensus	*Dd* ACA-2	*Dd* ACG
Dd cyclase	33%	19%	21%	27%

mismatch on amino acid level. In AG1 only two out of seven amino acids are identical. Theoretically a combination of primer AG2 and GC3'B would have had a good chance to yield the product as was presented here, however, since the length of the obtained PCR products did not agree with the expected length this PCR material was not subcloned and sequenced.

Each strategy as presented in the Results and Discussion section, employed to isolate the *D. discoideum* GC gene was unlikely to be successful in finding the gene as presented above. The small but significant deviations of the conserved regions in the catalytic domain in this gene compared with related genes led to fatal mismatches in the PCR and other primers. However, the homology of parts of the isolated gene fragment to conserved regions in known guanylyl cyclases makes it likely to be a guanylyl cyclase instead of an adenylyl cyclase.

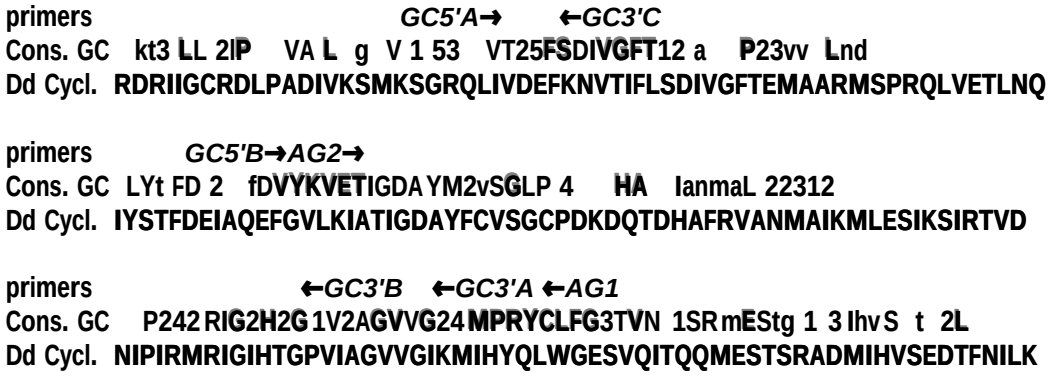

Figure 7. *Amino acid translation of part of the 1050-bp clone of the putative guanylyl cyclase gene from Dictyostelium and alignment with the guanylyl cyclase consensus sequence.*
In the consensus the conservative replacements are marked by a number that codes for homologous amino acids as follows 1=STPA, 2=MILV, 3=NDEQ,4=HRW, 5=FYW. In bold the residues that were conserved in all guanylyl cyclase. Uppercase characters show the residues that were conserved in at least 14 out of 17 sequences, lowercase are conserved aa's in at least 10 out of 17 sequences. Above the consensus sequence the positions of the PCR primers is given.

Summary

Summary

This thesis presents a number of studies of the molecular components that form the core of signal transduction in the social amoeba *Dictyostelium discoideum*. The concept of signal transduction comprises the passage of chemical messages from a cell's surroundings to its interior, chemical signalling from one side of the cellular interior to the other and spreading signals into the extracellular surroundings. Chapter 1 provides an overview of the molecular elements that play a role in signal transduction pathways in general.

The fascinating life cycle of *Dictyostelium* and its chemotactic behaviour towards cAMP and folic acid attracted the attention of scientists already in the thirties. The signal transduction network in *Dictyostelium discoideum* is studied as a model system, because this slime mould, being a lower eukaryote, is a relatively simple organism. In *Dictyostelium* signals are selected from the extracellular space by receivers or receptor molecules, located in the cell membrane. The receptors take care that the signal is spread in the cellular interior. Upon receptor stimulation with chemoattractant it is observed that the intracellular molecular composition changes and that molecules are secreted by the cell. On microscopic level, this cascade of molecular events results in pseudopod formation: movement of the cell towards the chemoattractant.

The cAMP receptor

The central molecule in the developmental stage of *Dictyostelium,* when the unicellular amoebae organize on waves of the chemoattractant cAMP, is the cAMP receptor. This membrane molecule, of which the synthesis increases when the food source becomes exhausted, enables the cell to sense the direction from where the cAMP molecules originate. An important feature of the receptor is that it becomes temporarily insensitive to its ligand, when the stimulating cAMP concentration remains constant or decreases. This process, called adaptation, enables the starving cells to establish the position of the cAMP producing neighbours, to move towards that direction and form a multicellular aggregate from which a next generation of amoebae will bud.

Adaptation of the cAMP receptor results in the temporary inability to activate intracellular responses. One way of communication of the receptor with its target enzymes occurs via a heterotrimer protein, the so-called G-protein. Conformational changes of the receptor molecule after prolonged stimulation is believed to disconnect this communication. The most likely way to achieve such a conformational change is by phosphorylation of particular amino acid residues in the receptor molecule. The cAMP receptor that was the first one to be cloned in *Dictyostelium,* cAR1, holds four clusters with a total of 18 serines that are plausible candidates for phosphorylation. Site directed mutations in this molecule revealed that two serine clusters were involved in phosphorylation upon receptor stimulation.

The cAMP mediated response of guanylyl cyclase and adenylyl cyclase in a mutant cell line CM1234, carrying a cAMP receptor in which all four serine clusters

were replaced or eliminated, is described in chapter 2. Wild type cells start producing cGMP within seconds after the cAMP receptor is bound to ligand, whereas the production of cAMP increases half a minute after receptor stimulation and peaks half a minute later. The observed time schedules of the responses in the mutant cell line CM1234 are not different from wild type responses.

The process of adaptation in wild type cells is reached also within seconds after receptor stimulation. Deadaptation, regaining responsiveness, is a process that takes place on a 5 - 10 minutes time-scale. Regarding the adaptation and the deadaptation of the cGMP response, the mutant cell line behaved similarly to cells with an intact cAR1 receptor. Also the adaptation process of adenylyl cyclase activation did not differ from that in wild type cells. Studying the deadaptation of the cAMP response revealed that the CM1234 receptor unlike its wild type counterpart, regained responsiveness quickly after removing the stimulus.

Based upon these observations we conclude that the adaptational and deadaptational events in the guanylyl and adenylyl cyclase responses are distinct processes. The cGMP response is not affected by removing the serines from the C-terminal part of the cAMP receptor. Adaptation of this response must occur at another element of the signal transduction cascade. Adenylyl cyclase behaves differently. Instead of an altered adaptation of the responses, due to the lack of serine phosphorylation, the adaptation of the CM1234 receptor functions completely normal. This means that adaptation cannot be the effect of phosphorylation of the C-terminal serines of the cAMP receptor. Instead the interaction of ligand and receptor itself seems to keep the receptor in a desensitized state. However when the stimulus is removed, the receptor regains its responsive conformation within 10 seconds, suggesting that C-terminal phospho-serines keep the receptor locked in a desensitized state. We present a receptor model in which the dephosphorylation of the adapted receptor is the rate limiting step in the adaptation of the cAMP response. In this model the conversion of the occupied adapted receptor via the adapted unoccupied receptor to a free susceptible receptor molecule occurs within 10 seconds.

The characteristics of Adenylyl Cyclase G

Dictyostelium possesses two genes that code for the protein adenylyl cyclase. The first one of the enzymes, ACA, is expressed early in the developmental stage, during aggregation. This enzyme is responsible for all the cAMP production during starvation, aggregation and fruiting body formation. The second enzyme, ACG, is produced not before the spores cells are formed, in the germination phase. By the predicted structure of this protein it was speculated that the protein would be a membrane bound guanylyl cyclase instead of an adenylyl cyclase. However, lysates of cells overexpressing ACG showed increased adenylyl cyclase activity compared to control cells, but normal guanylyl cyclase activity, proving ACG is a cAMP synthesizing enzyme.

The related ACA was studied intensively about a decade ago. In chapter 3 the study of the characteristics of ACG is presented. We observed that after stimulating the cAMP receptor, mutants that constitutively express ACG show an intracellular increase of its cAMP content, and one minute later the

extracellular cAMP also increases as well. This means that ACG must be coupled to the cAMP receptor. Further investigation showed that like ACA, the enzyme activity of ACG is inhibited by adenosine and caffeine. The enzyme activity of ACG is temperature dependent, and shows adaptation after stimulation of the cAMP receptor. When ACG is constitutively expressed in a cell line that lacks cAMP receptors 1 and 3, the activation of cAMP production does not occur, which means that one or both of these two receptors contribute to ACG activation.

Recently the protein CRAC, involved in the activation of ACA, was cloned. This protein transfers the signal of the stimulated receptor-G-protein complex to ACA. We showed that CRAC does not take part in the activation of ACG, by generating ACG overexpression in a *crac*$^-$ cell line, in which receptor stimulation of ACG still takes place. In cell lysates, ACA can be directly activated via the G-protein complex by the addition of GTPγS. In lysates of *aca*$^-$*/ACG* cells the addition of GTPγS did not affect the *in vitro* activity of ACG, an indication that transduction of receptor stimulation to ACG is not intermediated by G-protein interaction. Direct evidence for this suggestion was found in a cell line lacking the Gβ subunit and overexpressing ACG. Stimulation of the cAMP receptor resulted in activation of ACG activity, proving that, quite unusual, G-proteins are not involved in the receptor mediated activation of ACG. The mediation of the activating signal from receptor to ACG might be searched in the G-protein independent Ca^{2+}influx that occurs after receptor stimulation. However, evidence that ACG is Ca^{2+} sensitive could not be obtained. Finally ACG, structurally resembling membrane bound guanylyl cyclases, shares with guanylyl cyclase the ability to be activated by the folic acid receptor. All these aspects show that ACG is a curious cyclase, combining properties of both typical adenylyl cyclases and guanylyl cyclases.

The role of guanylyl cyclase

In *Dictyostelium,* guanylyl cyclase activation is related to pseudopod formation and therefore to cell motility. Vegetative cells respond to waves of folic acid, secreted by bacteria, enabling them to chase their food source. In the aggregative stage, the chemotactic sensitivity shifts towards cAMP, produced by the starving neighbouring cells, finally resulting in aggregation of the amoebae. The speed of pseudopod formation, within a few seconds, coincides with the observed cGMP increase inside the stimulated cell. Upon receptor stimulation guanylyl cyclase activity increases within a second. The receptor mediated cGMP signalling response is a combined action of the signal transduction elements. In contrast to many other guanylyl cyclases, the *Dictyostelium* protein is not activated by Ca^{2+} ions. In the first part of chapter 4 we describe our observations on the inhibition of GC by Ca^{2+} ions. This phenomenon was also reported for the guanylyl cyclase found in retinal rod cells from bovine eye. The sensitivity of the *Dictyostelium* GC is essential similar to the retinal rod protein, maximal inhibition occurs at a concentration of 1 µM Ca^{2+}.

Other elements that contribute to the receptor mediated cGMP increase and decrease are described in part II of this chapter. A detailed mathematical model of the molecular components that make up the *Dictyostelium* signal transduction process is presented. Cyclic GMP that

has accumulated after receptor stimulation is degraded by a cGMP specific phosphodiesterase, which is allosterically activated by its substrate. With the model we investigated the contribution of each structural and regulatory component in the cGMP response. Four different theoretical approaches on the conformational changes in the stimulated cAMP receptor are compared with experimental observations. Only one of these four follows the experimental observations in detail. The role of Ca^{2+} was investigated by changing the extracellular and intracellular Ca^{2+} concentrations. In the simulation experiments the effect of removing extracellular Ca^{2+} has a greater impact than was found in the chemical experiments. This indicates that the mechanism of receptor stimulated alteration of cytosolic Ca^{2+} is yet not fully understood.

Finally the cGMP response in mutant *stm*F, a cell line that is deficient in cGMP-specific phosphodiesterase, is analysed by removing the phosphodiesterase activity from the model. The model here predicts a cGMP response that is prolonged and increased, similar to the reported observations on the *stm*F cGMP response. From these simulation experiments we conclude that our model quite accurately describes the kinetic values of the events that are elicited by excitation of the cAMP receptor. It also indicates that the main structural and regulatory components of the cGMP response have been recognized.

Guanylyl cyclases are described in two structurally distinct forms: the soluble, cytosolic guanylyl cyclases, and the membrane bound insoluble proteins. The common motif in GC's is the catalytic domain is which conserved stretches can be distinguished, that show similarity to the related catalytic cores, present as a doublet in most adenylyl cyclases. In the last part of chapter 4, the strategies that were employed to clone the *Dictyostelium* GC gene are described. Based upon the similarities among the catalytic domains of previously published genes we designed oligonucleotide primers that were used in PCR. Though many fragments of genes were isolated, none of the segments could be identified as GC homologous. The screening of a *Dictyostelium* cDNA library was obstructed by the odd GC:AT ratio of *Dictyostelium* DNA compared with DNA of other species. We used a catalytic domain fragment of the membrane bound guanylyl cyclase from the sea urchin *Strongylocentrotus*, a lower eukaryote that like *Dictyostelium* has a low GC:AT ratio in its DNA. Under the investigated conditions no hybridization with *Dictyostelium* Southern blot was detected. Another approach using an oligonucleotide probe to hybridize with DNA on a Southern blot, gave clear bands, but screening the library resulted in the isolation of DNA fragments that had no similarity to the desired GC gene.

A total different strategy was trying to find the gene by screening for its product, cGMP, in an *E. coli* strain, that relied for its survival on the production of cGMP, transformed with a *Dictyostelium* expression library. This complementation system, though promising, appeared to be insufficiently selective.

The final approach in GC cloning, described in this thesis, was an immunological screen of an expression library. The antibody that we used was an oligopeptide designed after a conserved stretch in known guanylyl cyclases. This system identified a 53 kD protein band on an SDS-PAGE gel, but was not selective

enough to make clear discrimination between several positive and false positive colonies.

Recently our group succeeded in isolating a gene that exhibits all of the required features that makes a protein a guanylyl cyclase. With this success an end has come to a long lasting search. The future experiments should concentrate on a full description of the gene and the generation of overexpression, deletion mutants and point mutants. Another interesting field of investigation is elucidating the mechanism of Ca^{2+} inhibition of this protein. Possibly a Ca^{2+} binding protein can be isolated that is involved in the regulation of GC. With the cloning of this gene a new exciting phase in the study of *Dictyostelium* guanylyl cyclase has begun.

Nederlandse Samenvatting

Samenvatting

Levende wezens zijn in hun bestaan afhankelijk van communicatie, het afgeven en ontvangen van signalen. In biochemische zin kan signaaloverdracht worden omschreven als het doorgeven van chemische boodschappen van de omgeving van een cel (of organisme) naar het interne van de cel, chemische signalen van de ene kant van de cel naar de andere kant en uit verspreiden van chemische signalen naar de buitenwereld. Door middel van signaaloverdracht is een organisme in staat zijn leefomgeving waar te nemen en kan zo bijvoorbeeld voedsel en gevaar traceren.

De slijmschimmel *Dictyostelium discoideum*, studieobject in dit proefschrift, is een microörganisme dat de bodem van de Noordamerikaanse bossen als natuurlijke leefomgeving heeft. *Dictyostelium* heeft een fascinerende levenscyclus, waarin na een lange tijd van voedselopname en vermeerdering van het aantal cellen een metamorfose optreedt van het solitaire eencellige stadium naar een meercellig organisme. Onder gunstige omstandigheden leeft het organisme als amoebe, zich voedend met bacteriën, sporen van andere organismen en dood organisch materiaal. Wanneer de voedselvoorraad dreigt op te raken, is dit voor de slijmschimmel een aanzet tot de ontwikkeling naar het meercellige stadium, het vruchtlichaam, bestaande uit een hoofdje gevuld met sporen dat gedragen wordt door een steeltje, van een tot enkele millimeters groot (hoofdstuk 1, figuur 5).

De aanwezigheid van bacteriën wordt door de *Dictyostelium* amoeben waargenomen met behulp van receptoren, complexe molekulen die zich in de celmembraan bevinden, waaraan foliumzuur bindt dat uitgescheiden wordt door bacteriën (zie figuur 1, hoofdstuk 1). De cellen zijn in staat de positie van hun voedselbron te lokaliseren doordat in de richting van de bacteriën het aantal foliumzuurmolekulen toeneemt. De *Dictyostelium* cellen bewegen zich in de richting van de toenemende foliumzuurconcentratie, een proces dat chemotaxis genoemd wordt. Wanneer een amoebe gedurende enige tijd geen voedsel meer kan vinden, verdwijnt de gevoeligheid voor foliumzuur en in de cel wordt het signaalmolekuul cyclisch AMP (cAMP) gevormd. Deze stof wordt door de cel uitgescheiden en diffundeert in de omgeving. Naburige cellen nemen met hun cAMP-receptoren de stof waar en reageren hierop met het zich in de richting van de hongerende cel te bewegen. Bovendien beginnen deze cellen zelf ook cAMP te vormen en uit te scheiden. Op deze manier ontstaat er een aggregaat van enkele tienduizenden tot honderdduizend cellen vanwaaruit het vruchtlichaam zich ontwikkelt.

Cellen kunnen zich verplaatsen door het vormen van pseudopodia, uitstulpingen van de celmembraan. Dit gebeurt onder invloed van de intracellulaire signaalstof cyclisch GMP (cGMP). Stimulatie van de cAMP receptor heeft tot gevolg dat de aanmaak van cGMP in de cel bijna onmiddellijk stijgt. Het cGMP zorgt er op zijn beurt voor dat er bundeltjes ontstaan van het “spiermolekuul” myosine, die vorm geven aan de schijnvoetjes.

De opeenvolging van reakties als

gevolg van het binden van het ligand cAMP aan de receptor wordt het signaaloverdracht netwerk genoemd. In dit proefschrift worden de waarnemingen beschreven van experimenten, die er op gericht waren de werking te begrijpen van een drietal verschillende componenten van dit netwerk.

Een overzicht van de componenten van het signaaloverdracht mechanisme in eukaryote (kernhoudende) cellen wordt uiteengezet in het eerste hoofdstuk. De membraanreceptoren zijn binnenin de cel gekoppeld aan de G-eiwitten, die een schakeling vormen tussen de receptor en hun doel, de enzymen. De funkties en eigenschappen van de belangrijkste enzymen, de adenylyl cyclases, fosfolipase C en guanylyl cyclase worden behandeld. Zijdelings worden de schier oneindige groepen van kinases (fosfaatkoppelende enzymen) en fosfatases (fosfaatsplitsende enzymen) onder de aandacht gebracht. De inleiding besluit met een overzicht van het signaaloverdracht mechanisme in *Dictyostelium,* de huidige stand van zaken.

Een belangrijke eigenschap van de receptor is adaptatie, het tijdelijk ongevoelig worden voor cAMP na blootstelling aan een bepaalde concentratie cAMP. De geadapteerde receptor kan alleen nog geaktiveerd worden door een cAMP stimulus met een hogere concentratie dan de vorige. De oorzaak van adaptatie is mogelijk het verbreken van het contact tussen de receptor en het G-eiwit, bijvoorbeeld door vormverandering van de receptor. Het is bekend dat het koppelen van fosfaatgroepen aan een eiwit dit soort verandering teweeg kan brengen. In het gedeelte van de cAMP receptor dat binnenin de cel zit, komt een reeks serinegroepen voor die gefosforyleerd worden zodra er cAMP aan de receptor bindt. In hoofdstuk 2 wordt het onderzoek beschreven aan een mutante cellijn, die een cAMP receptor aanmaakt, waarin de serinegroepen verwijderd zijn. Hierbij is er gekeken welk effect het ontbreken van deze groepen heeft op de adaptatie van de cAMP en cGMP respons. Het blijkt dat de adaptatie en deadaptatie van de cGMP respons en de cAMP respons gescheiden processen zijn. Het ontbreken van fosforylatie van de receptor heeft geen invloed op de adaptatie en deadaptatie van de cGMP respons. Voor de cAMP respons is de conclusie genuanceerder. Naast een normale adaptatie van de cAMP produktie vertoonden de cellen een opvallend snel herstel van de gevoeligheid van de cAMP respons, wanneer de stimulus werd weggewassen van de cellen. Voor het adapteren van de cAMP respons moet er nog factor zijn, die tot op heden onbekend is. De fosfoserines zijn wel betrokken bij het vasthouden van de receptor in ongevoelige toestand, zolang deze gebonden is aan het ligand.

Cyclisch AMP wordt gesynthetiseerd door het enzym adenylyl cyclase. *Dictyostelium* bezit twee genen die elk coderen voor zo'n eiwit. Het eerste adenylyl cyclase (ACA) wordt aangemaakt vanaf het hongerende stadium en heeft zijn maximum in het begin van de ontwikkeling. Het tweede cyclase, ACG, wordt alleen gevonden in de sporecellen, in het laatste stadium van de ontwikkeling. Het ACA is betrokken bij alle cAMP-afhankelijke

ontwikkelingsprocessen met uitzondering van de sporevorming. De eigenschappen van dit eiwit zijn in de voorgaande jaren uitvoerig gerapporteerd. In hoofdstuk 3 wordt een uitgebreide studie gepresenteerd van de eigenschappen van ACG. Het molekuul onderscheidt zich in zijn bouw van andere adenylyl cyclases; het vertoont juist meer gelijkenis met de zogenaamde membraangebonden guanylyl cyclases. In de beschreven experimenten werd gebruikt van een mutante cellijn, met een uitgeschakeld ACA gen, waarin het ACG eiwit gedurende de hele levensyclus wordt aangemaakt. Stimulatie van de cAMP receptor veroorzaakte een toename van de cAMP concentratie in de cel en - even later- ook buiten de cel, een bewijs voor koppeling tussen de receptor en ACG. De waargenomen cAMP respons vertoont een adaptatie en deadaptatie lijkend op die van ACA. Het toevoegen van cafeine en adenosine aan receptorstimuli heeft een remmende werking op de aktivatie van ACG. In tegenstelling tot het eiwit ACA blijkt de enzymaktiviteit van ACG temperatuurgevoelig te zijn. De overdracht van het signaal van de gestimuleerde receptor naar aktivatie van ACG gebeurt zonder tussenkomst van G-eiwit en het eiwit CRAC, factoren die voor ACA-aktivatie juist onmisbaar zijn. Tenslotte blijkt dat het ACG geaktiveerd wordt wanneer de foliumzuurreceptor gestimuleerd wordt, een eigenschap die ook gevonden wordt bij het guanylyl cyclase. Het totaalbeeld dat van ACG verkregen is, toont een verrassend eiwit dat eigenschappen combineert die typerend zijn voor adenylyl en guanylyl cyclases.

Hoofdstuk 4 bevat een verzameling van studies die zich concentreren op het guanylyl cyclase. Dit enzym is verantwoordelijk voor de synthese van cGMP. Het toenemen en afnemen van de cGMP produktie is een ingewikkeld samenspel van factoren. Er is altijd wel een beetje aanmaak van cGMP, maar na stimulatie van de cAMP receptor neemt de produktie snel toe. De piek in de synthese ligt op 10 seconden na receptorstimulatie, een 30-45 seconden later is het enzym weer terug in rusttoestand. Het gevormde cGMP heeft maar een beperkte levensduur, omdat het spoedig wordt afgebroken door fosfodiësterases, enzymen die juist weer geaktiveerd worden door een toenemende hoeveelheid cGMP. In het eerste deel van het hoofdstuk wordt aangetoond dat bovendien Ca^{2+} een remmende werking heeft op *Dictyostelium* guanylyl cyclase.

In de loop der jaren zijn er veel experimentele gegevens verzameld over de regulatie van de cGMP repons. In het tweede deel van hoofdstuk 4 zijn deze gegevens samengebracht in een mathematisch model. Met behulp van computersimulaties wordt in een viertal wiskundige modellen van de cAMP receptor gekeken naar het effect van stimulatie met verschillende concentraties cAMP. De snelheid van cGMP vorming, het tijdstip en de hoogte van de piek en het terugkeren naar rusttoestand wordt vergeleken met de waarden die gevonden zijn in eerdere experimenten. Vervolgens worden simulaties gepresenteerd waarin de activiteit van het cGMP afbrekende fosfodiësterase wordt verkleind. De ontstane cGMP respons blijkt veel overeenkomst te vertonen met de

respons die waargenomen is bij een mutant waarin het fosfodiësterase ontbreekt. Tot slot wordt in een aantal simulaties de remmende invloed van Ca^{2+} ionen op guanylyl cyclase vergeleken met de resultaten van chemische experimenten.

In het laatste deel van het hoofdstuk wordt de zoektocht beschreven naar het gen dat codeert voor het guanylyl cyclase. In een groeiend aantal organismen werd de afgelopen tien jaar een of meerdere guanylyl cyclase genen geïsoleerd. Uit vergelijking van de aminozuurvolgorde van deze eiwitten blijkt dat er bepaalde motieven zijn die telkens terugkeren. Aan de hand van deze geconserveerde gebieden is met verschillende technieken geprobeerd (een deel van) het *Dictyostelium* guanylyl cyclase gen in handen te krijgen. Geen van de pogingen die hier beschreven zijn heeft het gewenste resultaat opgeleverd. Tijdens het schrijven van dit proefschrift zijn andere medewerkers van de vakgroep erin geslaagd een gen te isoleren dat op een aantal cruciale punten gelijkenis vertoont met andere guanylyl cyclase genen. In de appendix van het hoofdstuk wordt hierop ingegaan en aangetoond dat het gevonden gen dusdanig afwijkend is, dat het met de eerder gevolgde strategieën ook niet gevonden had kunnen worden.

Samenvattend kunnen we stellen dat de verschillende onderzoeken verklaringen hebben aangedragen voor de werking van het signaaloverdracht systeem in *Dictyostelium.* Het is echter ook duidelijk dat ons inzicht in de complexe materie nog lang niet volledig is. Met name de studie van het adaptatieproces van de cAMP receptor en de bevindingen van het pas gekloneerde guanylyl cyclase gen zullen in de toekomst interessante onderwerpen zijn.

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Stellingen

behorende bij het proefschrift

Regulation of adenylyl and guanylyl cyclase in *Dictyostelium discoideum*

Romi Valkema, 27 februari 1998

1. De bewering van Pitt *et al.* dat adenylyl cyclase activiteit in *aca*$^{-}$/ACG ongevoelig is voor cAMP stimulatie, is onjuist. (Pitt *et al.* (1992) Cell 69, 305-315)

2. Adenylyl en guanylyl cyclase(s) van *Dictyostelium discoideum* houden zich niet aan geijkte patronen. (Dit proefschrift)

3. Adolphe Sax had, in plaats van het ontwikkelen van de saxofoons, zich beter kunnen bezighouden met een uniforme naamgeving van zijn andere uitvinding, de saxhoorns.

4. Geheel in het licht van de door hem beoefende wetenschap kan het geval Diekstra correcter worden ondergebracht bij de “Freudiaanse verschrijvingen” in plaats van bij het “Plagiaat”.

5. Bij aselekte steekproeven voor statische studies van ziektebeelden wordt de derde wereld systematisch overgeslagen.

6. Onweersbuien die worden voorgesteld in films doen de werkelijkheid onrecht aan: in het echt bereikt het geluid van de donderslag de waarnemer ook wel eens niet gelijktijdig met de lichtflits van de bliksem.

7. Bij de gemiddelde weggebruiker is de wellevendheid in het verkeer omgekeerd evenredig aan het testosterongehalte

8. Wennen aan een nieuwe werkkring wordt vergemakkelijkt wanneer de meest gebruikte termen respectievelijk cyclic Guanosine Mono Phosphate en current Good Manufacturing Practice zijn, die vervolgens in de afgekorte vorm met cGMP worden aangeduid.

9. Bij het vergelijken van beroepsblaasorkesten met amateurblaasorkesten is steeds vaker de muzikale kwaliteit niet het onderscheid, maar juist het feit dat de eerste voor zijn kunstjes betaald wordt, terwijl de tweede voor hetzelfde geld moet bijleggen.

10. De herkenbaarheid van treinstations zou toenemen wanneer er, naast de bestaande parallel aan de rijrichting geplaatste stationsnaamborden-, een aanduidingssysteem zou zijn waarbij de naam van het gepasseerde station haaks op de rijrichting staat.

11. Met de wet op geregistreerd partnerschap wordt een nieuw soort van (legale) schijnhuwelijken geïntroduceerd.

12. Een fietsketting zal bij voorkeur van het kettingblad aflopen enkele dagen nadat deze ruim in het vet is gezet.

13. Katten zijn moordbeesten.